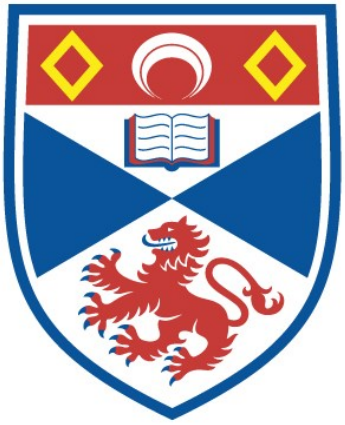
CHEMOTHERAPEUTIC RESPONSES OF MARINE MYELOID LEUKEMIAS

Aminu Abdussalam Abubakar

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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Aminu Abdussalam Abubakar

A thesis submitted for the degree of doctor of philosophy of the university of St. Andrews.

Department of Biology and Preclinical Medicine,
University of St.Andrews,
Scotland, U.K

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Declaration for the Degree of Ph.D.

I, Aminu Abdussalam Abubakar hereby certify that this thesis has been composed by myself, that it is a record of my own work, and that it has not been accepted in partial or complete fulfilment of any other degree or professional qualification.

Signed Date 7th March 1990

I was admitted to the Faculty of Science of the University of St Andrews under Ordinance General No 12 and as a candidate for the degree of Ph.D. on 5 January 1987.

Signed Date 7th March 1990

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate to the Degree of Ph.D.

Signature of Supervisor Date

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DEDICATION

I dedicate this thesis to my wife Karima and our two children Abdussalam and Nafisa, for their love and understanding.

ABSTRACT

The murine myeloid leukaemias employed in this study were induced in male CBA/H mice following irradiation with sublethal doses of X-ray. The responses of these leukaemic cell lines and normal (murine) bone marrow cells to cytosine arabinoside and mitoxantrone treatment in vitro were monitored. Both clonogenic, and nonclonogenic chemotherapeutic assays such as radioactive precursor uptake, dye-exclusion assay and autoradiography were employed to determine drug-induced cell lethality. In addition, the in vitro proliferative responses of the leukaemic cell lines and normal bone marrow cells to growth factors were determined using a [3H]thymidine uptake assay.

Both cytarabine and mitoxantrone were as toxic to normal bone marrow cells as to leukaemic cells from most of the cell lines. Mitoxantrone appears to be more potent than cytarabine against leukaemic cells in vitro. However, it was also more toxic to normal bone marrow cells. Generally, combinations of cytarabine and mitoxantrone resulted in an additive cytotoxic effect.

Mitoxantrone appears to have a narrow therapeutic margin when administered to leukaemia bearing mice in vivo. The response of the (SA7) myeloid leukaemic cell line to mitoxantrone was distinctly different from those reported for murine lymphoid leukaemias (P388 and L1210). Doubling the mitoxantrone dose within the therapeutic range was not accompanied by an increase in the number of long-term survivors (cures). Bone marrow cells of cured mice or normal(CBA/H) mice administered low doses of mitoxantrone became less sensitive to subsequent treatment with mitoxantrone in vitro . Doses of mitoxantrone that resulted in loss of protective effect by bone marrow cells of normal mice were toxic to leukaemia bearing mice.

Primary and low cell dose transplant myeloid leukaemias were less responsive to growth factors as compared to the high cell dose passages. The SA2 leukaemic cell line grew in vitro without requirement for growth factors. However, no growth was observed in serum-free medium which suggests that serum was providing the stimulus for in vitro proliferation.

Leukaemic bone marrow cells from the SA7 high cell dose passage cell line, were normally responsive to growth factors in vitro. However, at relapse following in vivo treatment with mitoxantrone, the leukaemic cells became significantly (P=0.04) growth factor insensitive. Bone marrow cells of normal mice retained growth factor sensitivity following in vivo treatment with mitoxantrone. Furthermore, bone marrow cells of mice cured of leukaemia by mitoxantrone treatment in vivo were responsive to growth factors.

Recovery of growth factor responsiveness occurred when the recurrent leukaemic cells were passaged in normal mice. However, no recovery of growth factor sensitivity was observed when recurrent leukaemic cells were passaged in mice that received a single dose of mitoxantrone (0.75mg/Kg) two days previously.

Even after passage in normal mice, the recurrent leukaemic cells were in some cases, significantly (P=0.012) resistant to mitoxantrone treatment in vitro. The degree of resistance appears to depend on the dose of mitoxantrone employed in the treatment of the leukaemia. However, passaging the recurrent leukaemia in mitoxantrone pretreated mice did not increase the level of resistance developed by the leukaemic cells.

These results suggest that these myeloid leukaemic cell lines may be useful models for preclinical evaluation of chemotherapeutic agents.

1. INTRODUCTION

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CHAPTER ONE

INTRODUCTION

Haemopoiesis is the process whereby all types of blood cells are formed. In normal humans, the process is tightly regulated so that as old cells are destroyed new ones are formed to replace them and no excess cells of any lineage are produced. However, in some neoplastic diseases of haematopoietic system, this normal control of proliferation is lost, thus leading to abnormal proliferation of a particular cell lineage . Leukaemias are one of the commonest neoplastic diseases of the haematopoietic system.

1.1 LEUKAEMIA

Leukaemia literally means white blood and the leukaemias are a group of diseases characterized by progressive proliferation of white blood cells. (Thompson, 1977). They can be subdivided into two major types depending on the line of white blood cells affected : lymphocytic and myeloid forms. Both acute and chronic forms of both lymphocytic and myeloid leukaemias occur in humans. Only the acute myeloid type will be discussed as it is the subject of the study being reported.

Acute Myeloid Leukaemia (AML): In acute myeloid leukaemia (AML) there is excessive proliferation and abnormal differentiation of immature myeloid cells in the bone marrow and blood which result in disruption of normal haemopoiesis (Griffin and Vellenga, 1987). It is believed to be as a result of malignant transformation of haematopoietic stem or progenitor cells accompanied by clonal proliferation and accumulation of the transformed cells. Although the pathogenesis of

leukaemic transformation is poorly understood, it is believed to be a multi-step process (Champlin and Gale, 1987). An apparent maturation block occurs early in the myeloid pathway, although some circulating granulocytes and other mature cells may be derived from the malignant clone.

By the time leukaemic symptoms are manifested as a result of disturbed haemopoiesis, the leukaemic burden could be as high as 10^9 to 10^{12} cells and there is considerable heterogeneity among patients in cellular phenotype, degree of cytogenetic abnormality and response to chemotherapy. Generally, however, there are excess number of leukaemic blasts in the bone marrow and some enter the blood and infiltrate other tissues. In a majority of cases, the bone marrow is hypercellular, although in 5-10% of cases it may be hypercellular. In up to 25% of patients, AML was preceded by a myelodysplastic preleukaemic syndrome (Koeffler and Golde, 1980). In these patients, normal haemopoiesis is suppressed by ill-defined mechanisms. In most patients, normal progenitors persist in the bone marrow that can restore haemopoiesis following effective antileukaemic therapy. In rare cases, in patients in complete remission, there may be evidence of clonal haemopoiesis suggesting either maturation of the leukaemic clone or restoration of a preleukaemic state (Champlin and Gale, 1987).

Etiology : Although the exact cause of acute myeloid leukaemia is not known, a number of factors have been implicated which acting singly or in combination may be responsible for causing the disease. These include:

Radiation: Radiation exposure has been known to result in acute leukaemia. The most conclusive evidence was obtained from data

concerning the role of irradiation in the production of leukaemia among survivors of the atomic bomb blasts in Hiroshima and Nagasaki. There was a sharp rise in both chronic myeloid leukaemia (CML) and AML which continued for nearly 20 years after exposure. Although there appears to be no threshold value of radiation dose and evidence of leukaemia, no leukaemogenic effects were seen at radiation doses below 50 rads (Ellison, 1982).

Exposure to Chemicals: All drugs implicated as being leukaemogenic were known to cause bone marrow depression and or aplasia. The only chemical with unequivocal relationship to AML is benzene (Aksoy, Eredem and Discol, 1974). Even seemingly trivial exposure has been followed by development of AML with or without development of clinically recognised steps of aplastic anaemia, other cytopenias, myelofibrosis or myeloid metaplasia. Other suspected leukaemogens include Chloramphenicol, phenylbutazone and arsenicals. AML was also preceded by the use of alkylating agents for treating conditions such as ovarian cancer, gastric cancer and Hodgkins disease (Ellison, 1982).

Genetic Factors: Chromosomal abnormalities alone may not be sufficient to induce AML except with the aid of some external factors such as drugs, virus or physical agents. Diseases that predispose the patients to subsequent development of AML include Down's Syndrome and Bloom's Syndrome (Ellison, 1982).

Viruses: Both RNA and DNA viruses have been known to cause neoplastic disease in animals. Some viral particles have been demonstrated by electron microscopy in samples from patients with leukaemia. In addition, reverse transcriptase characteristic of type C RNA oncogenic virus and portions of nucleotide sequences similar to

those in human oncogenic viruses have been isolated from human leukaemia cells. It has not yet been determined whether this represents a cause-effect relationship or simply an evidence of endogenous type C virus which are ordinary occult but are activated incidentally within the leukaemic cells and which share many attributes with oncogenic viruses (Ellison, 1982).

Clinical Manifestation: These are related to suppression of haemopoiesis and the functional consequences of tissue and organ infiltration and include anaemia, neutropenia, haemorrhage, infection and or fever.

Classification: Classification of acute myeloid leukaemia is made on the basis of morphology of bone marrow and peripheral blood cells in Romanovsky-stained films and supplemented by certain cytochemical reactions such as test for peroxidases, which myeloid blasts stain positively. Also sudan- black and tests for non-specific esterases are used. The clinical classification is based on guide-lines proposed by the French-American-British (FAB) study group (Bennet et al,1976). Using this guide-line, presence of granules in cytoplasm and auer rods indicate myeloid origin of the leukaemia as they do not occur in lymphoid leukaemias. The myeloid leukaemias are subdivided into six main types (M_1, M_2, M_3, M_4, M_5 and M_6) according to (a) the direction of differentiation along one or more cell lines (b) the degree of maturation of the cells. Thus M_1, M_2 and M_3 show predominantly granulocytic differentiation and differ from each other only in the degree and extent of granulocytic maturation; M_4 shows both granulocytic and monocytic differentiation, M_5 predominantly monocytic differentiation and M_6 predominantly erythroblastic differentiation (Bennet, et al, 1976).

Oncogenes: Proto-oncogenes are the normal cellular counterparts of retroviral transforming genes (Griffin and Vellenga, 1987). It is thought that the products of these genes in normal cells regulate cellular proliferation and differentiation. Whereas the exact functions of oncogenes are unknown, some appear to produce either growth factors or growth factor receptors. For example, C-fms gene product is homologous with the CSF-1 receptor (Sherr et al, 1983). It has been speculated that activation of some proto-oncogenes alters cell growth by causing inappropriate growth factor production, activation of receptor in the absence of a ligand or abnormal receptor - coupled signal transduction. It may be that activation of one or more oncogenes initiate human tumour formation and evolution of progressively more malignant subclone takes place through activation of yet other oncogenes. Although the exact mechanism governing the activation of proto-oncogenes is not yet known, one mechanism that is likely to be important in AML is chromosomal translocations or deletions (Croce, 1986).

1.2 Growth of Leukaemic cells In Vitro

Substantial data on the biology of leukaemia are derived from observations of leukaemic growth in vitro. Experimental evidence suggests that leukaemia occurs as a result of imbalance between self renewal, proliferation and differentiation. Leukaemia is therefore seen as clonal proliferation of tumour cells able to escape normal controls of proliferation and differentiation.

Primary AML exhibit heterogeneity when grown in culture. Only a small fraction of cells are actually capable of proliferating - usually less than 1% (Moore, William and Metcalf, 1973). While few AML cases appear to have the capacity to proliferate autonomously in culture, many cases appear to have the ability to augment their own proliferation by either producing colony stimulating factors (CSF) or secreting factors such interleukin -I (IL-I) which induce normal accessory cells to supply CSF (Griffin and Vellenga, 1987).

Leukaemic colony forming cells (AML-CFU) are identified by their ability to form colony of leukaemic blasts in semi solid media (Buick, Till and McCulloch, 1987). It is likely that AML-CFU may function as stem cells in vivo. Firstly, they are actively synthesizing DNA as demonstrated by thymidine suicide studies (Minden, Till and McCulloch, 1978). Secondly, replating experiments have shown that AML-CFU have limited self-renewal capacity. However, tertiary replating was low and more than 4-generations of colonies have not been observed. Although in a few cases AML cells can proliferate in vitro without the need for exogenously added growth factors, many leukaemias require the presence of growth factors in order to survive and proliferate in vitro (Young, Wagner and Griffin, 1987).

1.2.1 Colony Stimulating Factors

These are glycoproteins that stimulate the proliferation of both normal and leukaemic cells in vitro. Four major types have been described and include:

Interleukin-3 (IL-3, Multi- CSF): This factor stimulates the growth and differentiation of multipotential stem cells and of progenitor cells committed to all the non-lymphoid lineages. Thus, it stimulates the formation (from normal marrow) of granulocytes, macrophages, mixed granulocyte and macrophage colonies, erythroid, eosinophillic and megarkaryocytic colonies.

Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF): This factor stimulates the formation of granulocytes, macrophages and precursors of mixed granulocyte and macrophages in normal bone marrow cells (Walker et al 1985). Most cases of acute myeloid leukaemia (AML) respond to the addition of IL-3 and GM-CSF by an increase in proliferation and growth factor induced proliferative response is at least in part dependant on the recruitment of blast cells from the resting phase to the proliferative compartment. Autonomous proliferation of AML cells is believed to involve, in some cases, activation of GM-CSF gene, and probably one reason why leukaemic cells take over normal bone marrow cells is through increased growth advantage as a result of autonomous proliferation.

Granulocyte Colony Stimulating Factor (G-CSF): This factor not only stimulates the production of granulocytic colonies in normal bone marrow cells, but it has also been reported to stimulate the formation of mixed granulocyte-macrophage colonies (progeny of bipotential progenitors) and pure macrophage colonies at high concentrations.

Macrophage Colony Stimulating Factor (M-CSF): This polypeptide is only active as a dimer. When it is dissociated by urea-mercaptoethanol or acidic acetonitrite, the isomer was found to be no longer active. It stimulates almost exclusively the formation of macrophage colonies.

Many of these colony stimulating factors are present in various conditioned media such as placenta conditioned medium and conditioned medium from various tumour cell lines (Lowenberg , Swart and Hagemeiejer, 1980).

The ability of colony stimulating factors to exert their biological effect is linked to the expression of receptors specific for that factor on the surface of target cells. It has been established through receptor binding studies that each of the four colony stimulating factors bind to a unique receptor (Walker et al, 1985). In addition, the receptors show complete specificity in that they do not bind other growth factors.

One of the obstacles of treating leukaemia by chemotherapy is that leukaemic stem cells are not normally in cycle and are therefore difficult to kill by cycle-active drugs (Butturini and Gale, 1989). There is evidence that at least some leukaemic stem cells respond to growth and regulatory factors . It is possible therefore, to use these factors prior to initiating chemotherapy in order to increase the efficiency of many drugs such as cytosine arabinoside which are more effective against proliferating cells. However, there is the potential drawback that normal myeloid or lymphoid stem cells may also be stimulated and will therefore be killed. However, it may be an acceptable risk if treatment will result in cure (Butturini and Gale, 1989). In order for in vitro observations on the behaviour of leukaemic cells to be relevant, they must be shown to occur in vivo as well. Experimental murine tumours are usually employed in studying the in vivo biology of tumours. These may be more relevant to the clinical situation as compared to in vitro models such as continuous murine cell lines.

1.3 Acute Myeloid Leukaemia Models

Experimental myeloid leukaemia models are useful in the study of (a) leukaemogenesis (b) failure of normal haemopoiesis in leukaemias and (c) therapeutic procedures etc.

Leukaemia and lymphoma models have been used for screening new antitumour agents and the results in some cases have correlated well with the human disease. The vast majority of conventional cytotoxic agents have had their activity discovered against mouse leukaemia and lymphomas before being introduced into clinical trials and all compounds that are active against human leukaemia and lymphoma are effective against some strains of rodent lymphoma and leukaemia (Birchenal, 1982).

There has been a lot of controversy over the relevance of transplanted leukaemia as models of the disease in humans. According to Schofield (1982), they do not provide good models of human leukaemias, because the latter occur spontaneously. Differences between long transplanted syngeneic leukaemias; the spontaneously occurring leukaemias in AKR mice and the human disease were highlighted using a comparable chemotherapeutic study (Birchenal, 1982). Approximately 99% of the cells in L1210 transplantable leukaemia are in cycle (with cell a cycle time of 12 hours). When cytosine arabinoside which is a cell cycle specific agent was administered in a schedule that maintains a lethal concentration of the drug constantly in contact with the cells over two cell cycles followed by a 3-day interval to allow normal cells to recover, a net 5-6 log cell kill was achieved and a high percentage of mice were cured. On the other hand, when the same schedule was applied to AKR spontaneous leukaemia in which the cell cycle time was at least 24 hours and only 1% of the cells are in cycle, with a leukaemic burden of 10^9 cells,

no cures were seen. Similarly, in human acute lymphoblastic leukaemia, the cells may have a doubling time of 2-4 days in the early stages of the disease, but in the latter stages of the disease, doubling time as high as 27 days have been reported (Clarkson and Fried, 1971). This indicates that in late stage of the disease, very small number of cells are in cycle and therefore cycle-specific agents alone will be of little use.

Despite these drawbacks however, studies of combination therapy in mouse leukaemia have led to such active combinations as cytarabine and cyclophosphamide; cytarabine and thioguanine; vincristine and asparaginase etc (Gale and Cline, 1977). The treatment principles of intensive chemotherapy, schedule dependence, chemotherapeutic sanctuaries were all derived from mice models. These principles when applied in the clinic have resulted in an ever increasing percentage of 5-year survivors.

There are relatively few myeloid leukaemia models available and a survey of the literature reveals the following models:

WEHI-3: This is a myelomonocytic leukaemia that was induced in BALB/C mice following mineral oil (paraffin) injections. It is characterised by a mixed picture of early monocytic and myelocytic cells in contrast to pure granulocytic or pure monocytic forms of the disease. The supporting evidence for the cytological classification of WEHI-3 as a myelomonocytic leukaemia was the finding of high serum, urine and cellular levels of muramidase. This cell line also produce colony stimulating factors in vitro (Metcalf, Moore and Warner, 1969).

L5222: is an acute rat leukaemia which was induced as a result of administration of nitrosourea. On cytochemical and electron microscopical grounds it was classified as a myelomonocytic leukaemia.

After injection, the leukaemic cells proliferated mainly in the bone marrow and spleen and later they are detectable in the blood and also infiltrate other organs. A direct relationship was found between the number of cells injected and the time course of leukaemia development. When 10^7 leukaemic cells were injected the animals died within 12-13 days (Hoelzer, 1973).

BNML: This leukaemia was induced in Brown Norway (BN) rats using 9,10-dimethyl 1,2-benzanthracene. A reproducible growth pattern was observed upon transplantation. The model resembles very closely human AML (Hagenbeek and Martens, 1973). Cytological and biochemical studies have revealed that the leukaemia is promyelocytic with a slow growth rate (10^7 leukaemic cells kill after 28 days) and severe depression of normal haemopoiesis during leukaemia growth due to decreasing number of normal haematopoietic stem cells (Bekkum et al, 1976).

Sutton August Leukaemia (SAL): Although spontaneously occurring leukaemias in rats are uncommon, the SAL leukaemia arose spontaneously in normal female august rat and was maintained by serial transplantation through syngeneic rats. Rats died 8-10 days following implantation with 10^4 SAL leukaemic cells. The cause of death was anaemia and infection occurring one day before death. Bone marrow was taken over by grossly abnormal leukaemic blasts. The leukaemia was classified as myeloid on the basis of the following evidence:

- the leukaemic cells primarily colonise bone marrow and any lymphoid organ involvement was apparently of secondary nature.

- in the spleen, the leukaemic cells proliferated mainly in the red pulp and not in the malpighian corpuscles.

- lymph nodes were not extensively infiltrated and lymphoid follicles remained intact.

- despite a high percentage of blasts in the blood, very few were detected in the thoracic duct lymph.

- the leukaemic cells were not lysed by antithymocyte serum which is lytic for rat thymocytes (Wrathmel, 1976).

Shay Chloroleukaemia: Shay Chloroleukaemia was induced in rats following gastric instillation of 20-methylcholanthrene and was classified as acute myeloid leukaemia on the basis of morphological and cytochemical characteristics. There are three distinct forms:

When transplanted subcutaneously (SC), a local tumour developed; when transplanted intraperitoneally (I-P), both ascites tumour and a generalised leukaemia resulted; when the cells were injected intravenously (IV), a generalised disease was manifested. The main symptoms in terminally leukaemic mice were skin pallor, weakness and ruffled fur. When $2x10^7$ chloroleukaemic cells were injected, the mice died within 8 days and with each 10-fold reduction of Inoculum, survival was increased by about 2.5 days (Foa, 1981).

Radiation-Induced Myeloid Leukaemias: No spontaneously developing leukaemias were found in CBA/H mice (Major and Mole, 1978). This makes radiation-induced myeloid leukaemia a good model for the study

of the leukaemogenic process. In addition, the leukaemia does not appear to be immunogenic (Hepburn et al, 1987). The incidence of leukaemia in irradiated mice was about 20% at the optimum X-ray dose of 150-300 rads (Major and Mole 1978). If 600 rads were administered, 1, 4 or 32 weeks following 300 rads, myeloid leukaemia frequency was markedly reduced. The dose response was curvilinear with leukaemia incidence decreasing within the dose range of 400-600 rads.

Recently, other radiation induced myeloid leukaemia models were reported (Hepburn et al, 1987). The leukaemias were induced following 3 Gy whole-body X- irradiation in CBA/H mice. In terminally leukaemic mice, there was noticeable pallor of the feet, ruffled fur, weakness and loss of weight. The blood showed increased white cell counts accompanied by decreased haematocrit and haemoglobin counts. The spleen was massively enlarged and the bone marrow was replaced by leukaemic cells and the liver was often infiltrated as well. No lymph node involvement has been reported. It was possible to serially passage the leukaemic cells in syngeneic recipient. All mice receiving 10^6, 10^5 or 10^4 primary leukaemic cells died of leukaemia within 25-35 days. However, 40% of mice receiving 10^3 cells remained leukaemia free for an observation period of 120 days. After the leukaemia was passaged 17 times, it was possible to transfer the disease with as little as 10 cells (Hepburn et al 1987). Depending on the cell dose that was used to routinely passage the primary leukaemia, two distinct cell lines with different transplantation characteristics were obtained. All experiments reported in this study were performed using these models.

One of the most important uses of murine models is in the design of more effective treatment schedules for eradicating leukaemic disease in humans.

1.4 Treatment of Leukaemia in Humans: The general principles underlying treatment in humans is discussed (section 1.4.1) followed by the mechanism of myelosuppression (section 1.4.2) since this is the dose limiting toxicity. Next, is a discussion of the pharmacology of Ara-C and mitoxantrone (Section 1.4.3) the two drugs used in this study, followed by the principle behind drug scheduling (Section 1.4.4).

1.4.1 Principles of Treatment: Evidence from chemotherapy of acute leukaemia in both humans and animals indicate the presence of a dose-response relationship: that is higher doses produce more cures (Butturini and Gale, 1989). If this is true then why has increasingly intensive chemotherapy not cured more people with leukaemia? There are many reasons that have been suggested as responsible for the failure. These include the possibility that chemotherapy was not sufficiently intensive, or that inferior drugs or schedules are used. Another possibility is that treatment fails not only because of resistant leukaemia but because it is too toxic to normal bone marrow cells needed to restore haemopoiesis.

Several models have been proposed to explain the current results of intensive chemotherapy of leukaemia (Butturini and Gale, 1989). First, in a homogeneous population with chemotherapy resistant leukaemia, there would be no dose response relationship and no cures. In practice, this has never been obtained, since some people with leukaemia are cured and therefore the first model cannot be correct. In the second model, a homogeneous population is considered in which leukaemia in that population is responsive to chemotherapy. In this case, increasing the dose will result in increasing cure until all members of this population are cured. In the third model the population of people with leukaemia consists of two sub populations one in which (a) the

leukaemia is responsive to chemotherapy and (b) the leukaemia is resistant to chemotherapy. The population in (a) would show a dose-response relationship to chemotherapy, increasing doses will result in increasing cures until all cases in this subpopulation are cured. The non-responsive population (b) would not show any dose-response relationship and more intensive chemotherapy will not be accompanied with increasing cures. Thus, in the third model, more intensive chemotherapy produces more cures until a plateau is reached after which higher doses do not achieve more cures, and may even result in decreased outcome due to toxicity. It has been suggested that this model best explains current results of intensive chemotherapy of leukaemia although the real situation may even be more complex. (Butturini and Gale, 1989).

The aim of chemotherapy in leukaemia is to eradicate the malignant clone. Treatment is usually subdivided into several phases. In the first phase, termed remission induction, the aim is to reduce the number of leukaemic cells to below the level of detection and restore normal haemopoiesis. By definition, no leukaemic cells should be detected in complete remission; however in practice, in most patients, substantial numbers of malignant cells probably persist (Lowenberg and Hagenbeek, 1984). This ultimately leads to leukaemia relapse if effective post remission therapy is not administered. The goal of post-remission therapy is to eradicate minimal residual disease. Unfortunately, resistance to treatment commonly develops and most patients eventually relapse despite continued therapy.

The combination of cytarabine and an anthracycline has been the cornerstone of remission induction chemotherapy. The best results utilised Ara-C 100-200mg/m^2/d by constant or intermittent I.V. infusion for seven days in combination with daunorubicin 30-70mg/m^2/d by I.V.

bolus on the first and last 3 days (Gale, 1984). If remission is not induced following one or two courses of this regime, the patients have poor prognosis. Even if remission is ultimately achieved, it is typically brief (Champlin et al, 1984). In 50% of the treatment failures, the leukaemia may be drug resistant while in the other 50% the patients might have suffered from chemotherapy related toxicity such as infections, bleeding (Estey et al, 1982). Combinations of cytarabine with either amsacrine or mitoxantrone appears as effective as with daunorubicin (Shenkenberg and Von Hoff, 1986). Patients with therapy induced AML (as a result of treatment of other neoplastic conditions) have only a 20-40% likelihood of achieving remission with standard induction chemotherapy.

There is no convincing data that Ara-C administered in low doses is as effective as at conventional doses for induction chemotherapy in high-risk patients and its use is not recommended if the aim is to induce remission (Butturini and Gale, 1989).

The value of consolidation or early intensification, late intensification or maintenance chemotherapy in prolonging remission duration and in preventing relapse is controversial (Lewis, 1985).

In children, intensive chemotherapy results in about 35-50% 5-year leukaemia free survival (Grier et al, 1987). Results in adults with AML are less satisfactory with almost 25-35% long term leukaemia free survival (Gale and Foon, 1987).

Doses of antileukaemic drugs can be substantially increased if treatment is followed by bone marrow transplantation (BMT). BMT has been used as a form of consolidation treatment in order to induce remission in relapsed or resistant leukaemia . An advantage of bone marrow transplantation is that in many situations the relapse rate following allogeneic (HLA-identical) BMT is lower than following intensive antileukaemic therapy or even than following twin transplants

(Butturini and Gale, 1989). This is because several potential antileukaemic mechanisms may be operating. An example is the immune related anti- leukaemia effect mediated by lymphoid or other cells in the graft. There is also the added anti-leukaemia effect of conditioning regimen. Transplant-related immune mechanisms can be divided into three: (a) graft-versus host disease (GVHD) (b) graft-versus-leukaemia effect (GVL) and (c) an independent effect produced by T-cells independent of (a) and (b). It has been reported that these transplant-related immune mediated antileukaemic effect could be as effective as high-dose chemotherapy and radiation in eradicating leukaemia and offer a potential therapeutic option (Butturini and Gale, 1989).

Most tumour populations are inherently heterogeneous and they can undergo rapid phenotypic change and this represents two of the most formidable obstacles to effective antineoplastic therapies. More over, it has been reported that the treatments themselves can modify tumour cell phenotype and the rate at which variant cell subpopulations are generated. This is because most antineoplastic agents are mutagenic as well, but other non-mutagenic changes such as gene amplification are also implicated. The net result would be that cells that survive treatment and cause relapse may also possess an unstable phenotype (Lotan and Nicolson, 1988). Evidence suggests that random and spontaneous genetic changes not only result in development of drug resistant tumour types but could result in the acquisition of increased metastatic potential (Schimke, et al, 1986). As tumours progress and undergo phenotypic diversification they become less likely to succumb to therapy. In addition, treatment with chemotherapeutic agents have been shown to induce the metastatic phenotype (McMillan, Rao and Hart, 1986).

1.4.2 Myelosuppression: This is the principal dose-limiting toxicity of many cytotoxic agents. The latter can be broadly classified into three groups depending on the extent of myelosuppression they produce clinically. The first group typified by vincristine or L-asparaginase are rarely associated with bone marrow toxicity. In the second group are found drugs such as methotrexate, vinblastine and cisplatin, which cause mild to moderate myelosuppression. The third group of drugs are associated with predictable and potentially severe dose-limiting haematopoietic toxicity. Examples of this group include mitoxantrone and other anthraquinones; anthracyclines, cytarabine and other pyrimidine analogues, nitrosoureas and other alkylating agents (Gale, 1985).

While haematopoietic toxicity is produced rapidly by drugs such as hydroxyurea (within 2-3 days), with the pyrimidine analogues it takes 1-3 weeks. The severity of myelosuppression is dependent on the nature of the drug, its mechanism of action, the dose (total dose administered as well as dose per treatment), schedule of administration, route of administration, prior chemotherapy or radiation therapy and combination chemotherapy with other drugs (Gale, 1985). It is apparent therefore that both antineoplastic effect and myelosuppressive toxicity are dose-dependant. There is generally a narrow therapeutic margin and sometimes no margin in selective toxicity is seen between tumour cells and normal bone marrow cells.

Antineoplastic drugs produce myelosuppression through either direct or indirect effects. Direct effect refers to interactions of the drug or metabolite(s) with myeloid cells or their progenitors. The mechanism involved may include a direct toxic effect which may result in a proliferative defect or by causing an imbalance between self renewal and cell differentiation or maturation (Gale, 1985). The indirect effect of

antineoplastic agents is produced via interaction with the bone marrow micro- environment or by affecting haematopoietic growth or other regulatory factors. Examples of these mechanisms are provided by busulphan and cisplatin. While busulphan is directly toxic to bone marrow stem cells, cisplatin may cause anaemia by interfering with the renal release of erythropoietin. Similarly, the coagulation cascade may be activated following treatment of acute promyelocytic leukaemia with antineoplastic agents. This is as a result of the release of proagulant activity from the leukaemic promyelocytes and it could lead to disseminated intravascular coagulation (DIC).

Antineoplastic agents cause either intermediate, early, delayed or latent bone marrow suppression depending on their type. Drugs such as hydroxyurea and prednisone cause rapid reduction in blood cells detectable within 12-48 hours. Agents that cause reduction only after 1-3 weeks following drug administration may do so due to an effect on immature haematopoietic cells that only becomes apparent after mature cells die and need to be replaced. Granulocytes have the shortest life span (24-48 hours) and therefore, granulocytopoienia may be the first detectable effect of antineoplastic drugs. The next cell lineage to be affected are platelets (life-span 10 days) followed by red blood cells (life-span 120 days) and lymphocytes (life-span of many months).

It is uncommon for drugs like nitrosoureas and mitomycin C to cause delayed myelosuppression occurring 4-8 weeks after drug administration. The delayed type of myelosuppression is thought to selectively affect haematopoietic stem cells and requires several weeks to become evident. This latent effect has been studied primarily in mice treated with busulphan. Under appropriate experimental conditions, such mice have normal circulating blood cells but have markedly decreased number of myeloid stem cells. It is believed that the

pathophysiology of this effect is complicated but may relate to busulphan induced damage to stem cells, the bone marrow microenvironment and probably to lymphoid cells too (Gale, 1985). That the microenvironment is affected could be seen from the fact that transplantation fails to restore normal haemopoiesis in busulphan treated mice.

Although the exact mechanisms through which antineoplastic drugs affect haemopoiesis are subject to investigation, they may be unrelated to the mechanism of tumour kill produced by these drugs. This is demonstrated by the observation that cyclophosphamide which is a potent alkylating agent, is thought to have a selective effect on mature progenitors while sparing multipotent stem cells.

A number of strategies have been devised to minimise the impact of myelosuppression. The suggested methods include rescue using cryopreserved autologous bone marrow cells, enclosing drugs in liposomes as well as using antibody-directed drugs and regional chemotherapy (Gale, 1985).

1.4.3 Cytosine Arabinoside (Cytarabine, Ara-C): Ara-C is the principal drug of choice in the treatment of acute myeloid leukaemia (Ellison et al, 1968) and when used singly produces about 25% incidence of complete remission (Heidelberger, 1982).

Ara-C is an analogue of deoxycytidine and its mechanism of action is believed to involve inhibition of DNA polymerase and it is also incorporated into DNA and RNA. The degree of sensitivity of leukaemic cells to Ara-C is dependent on the relative activity of deoxycytidine kinase which converts the drug to the active nucleotide Ara-CTP. The latter is a potent inhibitor of DNA synthesis (Wist, Krokan and Pyrdz, 1976). Almost complete inhibition of DNA synthesis (79%) is required for Ara-C to be toxic (Bhuyan et al, 1973). The

Ara-C moiety are incorporated onto the ends of growing strands of DNA. Addition of the next deoxynucleotide is then inhibited and thus DNA replication is blocked (Kufe et al, 1980). An unusual finding was that following drug removal after temporarily blocking DNA replication forks with Ara-C, DNA replication is reinitiated in some segments of the chromosomal DNA that had already been replicated (Woodcock and Cooper, 1979; Woodcock, 1987). The consequence of this is that DNA segments are replicated more than once in a single cell cycle. This has been implicated as a probable mechanism of drug-induced gene amplification (Schimke, 1984). Ara-C is rapidly deaminated in many tissues to ara-uracil (ara-U) which is biologically inert. Since Ara-C is effective only in S-phase and is rapidly deaminated, in order to be maximally effective, it should be administered in frequent 12-hour pulses or continuous intravenous (I.V) infusion.

When Ara-C is given as a single agent by continuous I.V or 12-hourly injection, remission is achieved in about 20-30% of cases. The combination of one-day daunorubicin plus 3-5 days of cytarabine produces remission in up to 50% of patients. The prognosis for AML resistant to anthracyclines and Ara-C is poor.

The side effects encountered include nausea, vomiting, bone marrow suppression with very marked megaloblastic change. Less frequent side effects are fever, rashes and arthralgia.

Mitoxantrone (Mitozantrone, Novantrone(R)): Mitoxantrone is an anthracenedione which was synthesized in the 1970's (Murdock et al, 1979). It is structurally related to doxorubicin (Adriamycin) although it lacks an amino sugar moiety (Johnson et al, 1979). It inhibits both RNA and DNA synthesis and is reportedly 6-7 times more potent than doxorubicin in inhibiting [3H]-thymidine incorporation into DNA and

[3H]-uridine incorporation into RNA. Just like doxorubicin, it binds DNA by intercalation (Traganos et al, 1980). Cells in culture treated with mitoxantrone show nuclear morphological changes of chromosomal scattering and micronuclei formation (Murray and Wallace, 1980). Cytotoxicity was correlated with the extent of DNA strand breaks induced by mitoxantrone. It not only induces non-protein associated breaks in single stranded DNA, but it is also reported to act through non-intercalative electrostatic interactions. Mitoxantrone blocks cells in G2 phase (Traganos et al, 1980) and may be more effective in late-S-phase. However, cell killing induced by mitoxantrone is not cell cycle specific (Evenson et al, 1979). Furthermore, mitoxantrone is lethal to both proliferating and non-proliferating cells in vitro, which suggests potential activity in slow growing tumours. (Wallace et al, 1979). For example, in concentrations of 0.5ug/ml more than 90% of colon carcinoma cells incubated with mitoxantrone were killed regardless of whether they were dividing or not (Wallace et al, 1979). Mitoxantrone has considerable activity against various experimental mouse tumours. When administered I.P to mice bearing P388 leukaemia, it was more effective than doxorubicin in almost every treatment schedule. There was significant increase in life-span and high frequency of "cures" (60-day survivors).

Mitoxantrone is rapidly taken up by tissues and stored in several body compartments and gradually released. This is supported by its high volume of distribution (Savaray, et al, 1982). It is indicated in the treatment of acute leukaemia and relapsed cases seem to respond better than refractory cases (Masaoka, et al, 1985). When used alone, up to 20% of patients achieve complete remission (Champlin and Gale 1987). Response rate data in leukaemic patients previously treated and now in relapse or refractory with doxorubicin or daunorubicin support the idea

of incomplete cross-resistance between anthracyclines and mitoxantrone in acute leukaemia (Poirier, 1986).

Mitoxantrone is less cardiotoxic as compared to doxorubicin. Other toxicities in animals receiving mitoxantrone include effects on gastrointestinal tract, lymphoid system and bone marrow (Murray and Wallace, 1980). Cytogenetic analysis of bone marrow from rats treated with mitoxantrone showed increased chromosomal aberrations similar to those caused by other antineoplastic agents (Dulak et al, 1982). Mitoxantrone can also inhibit lipid peroxidation, a mechanism thought to be responsible for anthracycline induced cardiomyopathy. It also inhibits prostaglandin synthesis. Myelosuppression, predominantly granulocytopenia is the dose-limiting toxicity (Von Hoff et al, 1980). Myelosuppression is related not only to the amount of prior therapy but also to the extent of bone marrow involvement with tumour (Posner et al, 1985). Some deaths have occurred in granulocytopoienic patients secondary to infections and thus peripheral blood counts need to be closely monitored during therapy. Erythrocytes are not acutely affected although mild anaemia develops in most patients with successive courses of treatment. Thrombocytopoienia is less frequent. Other reported side effects include nausea and vomiting and diarrhoea (Poirier, 1986).

1.4.4 Scheduling of Drug Combinations: When two cancer chemotherapeutic agents are combined, a variety of drug-drug interactions ranging from antagonism to synergism can occur. For example, the use of Ara-C and asparaginase in combination chemotherapy of acute leukaemia is a logical choice since both agents are individually effective and have different mechanisms of action and host toxicities (Capizzi and Handschumacher, 1982). In practice, it was

observed that the combination of Ara-C and asparaginase was more effective than either drug alone in the treatment of human and murine leukaemia (Ekert, Colebatch and Mathews, 1972). Similarly, in the treatment of L5178Y leukaemia, whereas 3 doses of Ara-C administered on days 1, 4 and 7 after tumour implantation cured none of the mice, 3 doses of asparaginase alone cured 3 of 15 mice. In contrast, simultaneous administration of both drugs on each of the three days cured 36 of 43 mice (Schwart, Morgenstern and Capizzi, 1982).

Thus, the schedule in which cytotoxic drugs are administered could determine therapeutic outcome in both experimental and clinical settings. While some of the effects may be explained in terms of alteration of cell cycle kinetics, the mechanism underlying many of the interactions are yet to be elucidated.

1.5 In Vitro Chemotherapeutic Assays

For ethical reasons drug testing cannot be done in humans, and therefore, there is a need to devise appropriate preclinical models that would accurately predict response in humans. Unfortunately, no non-human assay predicts for efficacy in humans perfectly.

There are many factors that could influence the accuracy of an in vitro drug assay such as differences in cell kinetics in vitro and in vivo, altered chemosensitivity as a result of cells being removed from their normal environment and the in vitro culture method may select different subpopulation of cells. Also the lack of cell-cell contact as a result of preparation of a single-cell suspension, the in vitro drug concentrations and exposure times employed are important (Weisenthal,1981).

As mentioned, the choice of in vitro drug concentration is important. When doses are expressed in mg/m^2, there is a qualitative similarity between the dose of drug that will be lethal to 10% of mice (LD_{10}) and

the maximum tolerated dose in humans. As a result of this, most phase one trials in humans start at 10% of the LD_{10} in mice (mg/m^2). Traditionally, the concentration of a drug that has been used in in vitro assays has been 10% of peak plasma level. However, the actual concentration on the tumour bed (in-vivo) may be much lower or higher (Lock and Hill, 1988). In addition, peak plasma levels may not be satisfactory guides for those drugs that require extracellular or intracellular activation (Colins, 1988). Another parameter commonly used is the concentration x time (CXT) values. Results from in vitro cell culture experiments and some mouse tumour models system suggest that antitumour drug CXT is directly related to the degree of its cellular lethal effects (Alberts and Wetters, 1976). Thus, a range of clinically achievable drug concentrations should be used when selecting appropriate concentrations for sensitivity testing in vitro. In some experiments, an incubation time of one hour was chosen because pharmacokinetic data for intravenously administered drugs suggest that significant exposure to most drugs is greatest during the first hour after drug administration (Alberts et al, 1981). It has been suggested (Metcalfe, 1983) that concentrations lower than those clinically achievable should be used, since (solid) tumour drug levels may be less than that in the plasma due to poor perfusion within the tumour leading to loss of accumulation of the drug. Conversely, some drugs may be retained in tumours resulting in accumulation within the tumours for long periods. Drug resistant tumours arising from pretreatment in the patients, may need higher drug concentrations for testing in vitro.

Another problem with in vitro drug assays is that they do not take into account differences in host conversion and detoxification reactions and also tissue specific drug accumulation. It is also difficult to achieve the same total exposure to active drug metabolite in vitro as is produced in

vivo. In vitro end points of cell death may not accurately reflect in vivo kill and solid tumours present a particularly difficult problem of assessment (Weisenthal and Lippman, 1985). Drug induced cell lethality may be as a result of either metabolic death or reproductive death. Metabolic death is an event that can be measured by a variety of simple techniques that reflect immediate metabolic dysfunction, in contrast to reproductive death which is due to loss of proliferative capacity and is usually a delayed process (Roper and Drewinko, 1976). In proliferating cell populations, the inability to reproduce may be a more relevant assessment of cell kill. On the other hand, cells rendered incapable of sustained proliferation may still possess intact metabolic apparatus and may complete several cell divisions before the progeny perishes from the inherited damage. This means that assays of metabolic death may grossly over-estimate or under-estimate cell viability. In addition, some chemotherapeutic agents such as bleomycin may induce progression delay (Tobey, 1972). This results in a temporary lag in the multiplication rate and increases the doubling time of the treated cell population in comparison to that of control population. This may mimic the effect produced as a result of cell kill.

Wherever possible, a battery of tests rather than any single assay type should be employed. This is because no methodology exists which does not have its shortcomings. In addition, patients with the same histologic type of cancer do not respond uniformly to antineoplastic agents. And it has been suggested that empiric drug screening may be more fruitful if carried out in fresh specimens of human tumour rather than in established cell lines or non-human tumour systems. And in drug development, appropriate in vitro models could be used to test different treatment modalities in the laboratory thus improving efficacy of new drugs. An ideal in vitro test should be simple, statistically acceptable,

easily standardized, cheap, rapid, flexible and capable of taking account of different methods of drug action and should offer reasonable correlations with in vivo effects (Hamburger, 1981). The majority of in vitro chemosensitivity assays can be sub-divided into those assessing cellular damage such as loss of membrane integrity (dye-exclusion); measurement of inhibition of cellular metabolism; inhibition of radioactive precursor incorporation and lastly, measurement of clonogenic cells in a tumour cell population.

1.5.1 Dye-Exclusion or Differential Staining Cytotoxicity (DiSC) Assay:
This assay measures the uptake of dye by cells as they lose membrane functions following drug treatment, and are regarded dead by this criteria. While no direct proof was obtained that the dye (fast green) specifically stain dead cells only, the estimates of cell viability obtained with traditional trypan blue wet preparations and with fast green wet preparations were similar (Weisenthal et al, 1983a). As compared to cloning assays, the dye-exclusion assay requires 1/10th the number of cells. In addition, the assay is capable of detecting cell-kill in the non-dividing cell fraction and may therefore have unique advantages in new drug screening particularly for agents capable of killing cells in G_0. It may also be valuable for testing neoplasms which proliferate poorly in culture. Many cytotoxic drugs are not only effective against dividing cells but also alter essential cellular function in the resting phase of the cell cycle (Weisenthal and Lippman, 1985). Clonogenic assays do not measure such damage. Furthermore, clinical correlations have been obtained with dye-exclusion assays (Weisenthal and Lippman, 1985; Bird et al ,1986; Durkin, Ghanta and Miromoto, 1983).
Some of the shortcomings of the dye-exclusion assay include the fact that it is not an automated technique and is therefore relatively tedious and

subject to observer error. In addition, sufficient time must elapse following drug treatment for lethally damaged cells to lose their membrane integrity. The cells that survive drug treatment may proliferate during the time required for the lethally damaged cells to lose membrane integrity. A way must be found to account for lethally damaged cells that disintegrate before they are stained with the dye. The problems of proliferation in culture and disintegration of lethally damaged cells were solved by incorporating an internal standard of duck red cells in the assay; such that viability is assessed by determining the ratio of live tumour cells in drug treated samples in relation to control while relating both to the number of duck red cells (Weisenthal, 1983b; Bird et al, 1986). However, it may be difficult to differentiate tumour from non-tumour cells. In a study comparing in vitro effects of adriamycin, BCNU, vinblastine and cisplatinum on seven human tumours using a dye-exclusion assay a close correlation was found with in vivo results of drug therapy in all patients (Durkin, Ghanta and Hiramoto, 1983).

1.5.2 Tetrazolium (MTT) Assay: Tetrazolium identifies living cells by means of changing colour as a result of metabolic activity within the cells. The assay is a semi-automated colorimetric technique based on the principle that mitochondria of living cells reduce the tetrazolium salt (MTT) to formazan, a blue dye which can be read spectrophotometrically (Alley and Leber, 1984). As with the dye-exclusion assay, the MTT assay was found to be simple, rapid, inexpensive and reproducible. The assay has been used to determine the sensitivity of cytotoxic drugs in patients with acute myeloid leukaemia (AML) (Sargent and Taylor, 1989). Blast cells were exposed to single and combinations of drugs and their survival measured using the assay. A linear relationship was found

between the number of leukaemic cells and the optical density of the formazan produced. The assay was not only reproducible but was sensitive enough to detect variation in drug sensitivity between patients. No significant difference in sensitivity was observed between blast in the bone marrow and those circulating in the blood. Preliminary results show correlation between in vitro and in vivo data which suggests that the assay can be used to monitor changes in tumour sensitivity which could yield useful information that will assist in the management of acute myeloid leukaemia.

A drawback of the assay is that activated cells produced more formazan than resting cells (Mosman, 1973). Some cells do not produce sufficient amounts of formazan resulting in an insignificant difference in optical density between control and experimental groups. Furthermore, bacteria cleave tetrazolium salts to formazan. Therefore, false negative results may occur if visually undetected bacteria were present.

1.5.3 Assays of Inhibition of Cellular Metabolism: Direct measurement of respiration and glycolysis and measurements of cellular dehydrogenase activity shortly following in vitro drug exposure have been correlated with clinical response (Dipaolo, 1971). A potential problem is that this technique may not differentiate cells that are capable of repairing non-lethal damage from those that are reproductively dead. Additionally, the assay may not distinguish between tumour cells and stromal cells present in the preparation. Also, the test is incapable of differentiating damage to clonogenic cells and those cells that are non-dividing which make up the bulk of the tumour. However, an advantage of such assays is that they do not require successful growth of the tumour in culture to be interpreted. They are also fast, simple and may not require disaggregation into single cell suspensions (Weisenthal

and Lippman, 1985). Assays of resistance loci (Weisenthal and Lippman, 1985) also fall into this category. Resistance loci are factors responsible for resistance of tumour to damage produced by antitumour drugs. These include assays of the levels of uridine kinase and phosphoribosyl transferase (Ardalan et al 1981) which activates 5-fluorouracil as well as deoxycytidine kinase which activates cytosine arabinoside (Harris and Grahame-Smith, 1982); assays of cytidine deaminase which inactivates cytarabine (Smyth, Robins and Leese, 1976) and assays of methotrexate uptake into cells (Bender et al, 1976). Except for an encouraging correlation with 5-FU activation in patients with breast cancer, many of these assays have proved disappointing in clinical trials.

Unless the mechanism underlying drug resistance is known for most drugs, it is difficult for the resistant loci approach to gain wide acceptance. In addition, the test is not easily adaptable to generalised screening of drugs, since it would require a different type of test for each drug being screened. As knowledge of resistance mechanism expands, assays of resistance loci may find clinical applications (Weisenthal and Lippman, 1985).

1.5.4 Inhibition of Radioactive Precursor Incorporation: This assay measures the incorporation of radioactive precursors ([3H]-thymidine or [3H]-uridine) by proliferating cells in culture. In a typical assay (Raich, 1978), leukaemic cells were incubated in vitro with concentrations of Ara-C well below that clinically achievable (Alberts and Chen, 1980). After 36 hours incubation, [3H]-thymidine was added and the cells were labelled for 8 hours. Uptakes were measured in drug treated sample and related to control values. It was possible to correlate in vitro results with clinical response in patients with acute lymphocytic leukaemia, acute myeloid leukaemia, acute myelomonocytic leukaemia, acute monocytic

leukaemia and blast crisis of chronic myelocytic leukaemia (BC-CML). For many drugs, plots of [3H]-uridine incorporation resembled the respective plots of [3H]-thymidine incorporation. The standard deviation around the mean values for each concentration of a drug indicated a broad variability of in vitro response among individual tumours of the same type (Silvestrini, Sanfilippo and Daidone, 1983). It was suggested that it is best to delay labelling for several generation times after drug exposure in order to reduce problems of artifactual alterations in nucleoside intracellular pool sizes (Nakata and Bader, 1969) and salvage versus de novo dTMP synthesis pathway (Wolberg, 1972).

Unlike clonogenic assays, radioactive precursor uptake assays measure the drug effect on the whole cell population. There is evidence that very early disappearance of the entire leukaemic blast population correlates well with eventual sustained remission (Hidemann et al, 1982). Similarly, a study of response of human tumour xenograft to radiation and chemotherapy indicate that it is the effect on the whole tumour cell population that correlated with response (Weisenthal and Lippman, 1985). The radioactive precursor assay was able to detect drug resistant cells when such cells constituted only 10% of the total cell population (Bech-Hansen et al , 1977).

1.5.5 Clonogenic Assays: Whereas the dye-exclusion assay measures cell kill in the whole tumour population, clonogenic assays measure the degree of cell kill in dividing cells. These dividing cells constitute only a small proportion of the tumour population but are considered to be important because they are capable of repopulating the tumour. Single cell suspensions of tumour cells are plated into tissue culture plates or in semi-solid media in order to observe the number of colonies formed. The semi-solid conditions of the media most commonly employed are

agar or methylcellulose and they allow the progeny to remain localized and the number of colonies formed provides a minimum estimate of progenitor frequency (Hamburger and Salmon, 1977). In general, cells are plated at relatively low density (Rosenblaum et al , 1981) and are allowed to grow for 4-6 doubling times until colonies of greater than 50 cells are formed. The difference between drug treated and control cultures are presumed to relate directly to the fractional kill of clonogenic cells. The stem cell model of tumour growth is based on the principle that human tumours consist of a mixture of dividing and non-dividing cells (Tannock, 1978). In most cases, the bulk of the tumour cells turns over slowly. Of the non-dividing cells, some are irreversibly non-dividing (or permanent G_0) while others are reversibly non-dividing (temporary G_0) (Steel, 1977). Of the dividing cells, some are thought to have only a limited proliferative potential, while others are believed to have unlimited proliferative potential. The latter have been termed "tumour stem cells" (Steel, 1977). The end cells (those that have lost the capacity to divide) are lost either by limited differentiation, exfoliation or death. This model assumes that nonclonogenic in vitro assays may be measuring effect of drugs on cells that will probably not divide in vivo. The model assumes that only assays that measure effects of drugs on that small proportion of cells that maintain the growth of the tumour are relevant. Weisenthal and Lippman (1985) have proposed that to accept the validity of clonogenic assays, it is necessary to assume that (a) clonogenic cells in vitro are representative of stem cells in vivo, and (b) the stem cell population is the only relevant tumour cell target for chemotherapy. There are some reports in the literature suggesting the validity of the first assumption. For example, Mattox and Von Hoff (1980), have stated that colony formation is the only reliable indicator of cell viability following exposure to antineoplastic drugs.

Similarly, Chang (1983), has suggested that the colony forming assay should be the standard by which other short-term assays are to be compared, while Selby, Buick and Tannock (1983), have stated that clonogenic assays are the only assays which predict tumour cure rather than merely predicting partial response. In addition, there is evidence from work on animal and human tumours which seem to suggest that cells that are clonogenic under test conditions in vitro may be the same cells that are stem cells in vivo (Weisenthal and Lippman, 1985). Furthermore, using the clonogenic assay, in vitro resistance correlated with clinical resistance in 85% of patients whereas in vitro sensitivity correlated with in vivo sensitivity in 60% of the patients (Von Hoff et al, 1983B).

Other possible uses of clonogenic assays include tailoring of individual chemotherapy regimen for each patient as well as identification of patterns of chemosensitivity for patients with unknown primary carcinomas (Hill, 1984). The assay could prove useful both in screening of new antineoplastic agents and in in vitro phase II studies of those agents. And the assay could not only be used to monitor development of resistance, but it could also be of assistance in establishing patterns of cross-resistance and sensitivity in relapsing patients. Lastly, the assay could provide information regarding the relationship, if any, between clonogenicity and prognosis in patients (Hill, 1984).

1.5.6 Assessment of Cell Number: Holmes and Little (1974) described a microtitration test in which cells were treated for 72 hours with drugs before being tryptinized and counted with a coulter counter. A 40% reduction in cell numbers compared to control cultures was considered a significant in vitro response. In 11 out of 12 patients response was accurately predicted.

1.6 In Vivo Chemotherapeutic Assays

There is apparently less variety in the number of in vivo assays as compared to in vitro assays. A survey of the literature reveals the following:

Assessment of Increase in Life Span: Increase in life-span of tumour-bearing hosts is one of the commonest parameters for determining the effects of potential chemotherapeutic agents in vivo. Ideally, it should only be employed in studying effect of cytotoxic agents on the leukaemias (Valeriote, 1979) as solid tumours can have extremely wide distributions of time to death. However, even in leukaemias the assay is of limited sensitivity (Wilcox et al, 1966) in that such measurements assess clonogenic cells indirectly, i.e. after they have multiplied sufficiently to kill the host. Furthermore, in treated animals the assay may be misleading in the sense that it is unable to give adequate information on leukaemia sanctuaries such as the central nervous system and in immunogenic tumours there will be the added complication of tumour immunity which may lead to difficulties in data interpretation (Grindley, 1972).

1.6.2. Human Tumour Xenograft: In the human tumour xenograft model, the primary parameter of response is the extent of tumour growth inhibition as compared to controls. The treatment is started only after the tumours are well established and palpable at the site of implantation. Because of the relatively slow growth of the human tumour xenograft at the subcutaneous site of inoculation, tests may take as long as 60-90 days to complete. This assay utilizes heterotransplants of human tumours in the congenitally athymic "nude" (nu/nu) mouse. In a typical experiment, each mouse is inoculated subcutaneously (S.C) with up to 2×10^6 cells. When palpable tumours appear, control mice were

given sterile saline intraperitoneally (I.P) while test mice were administered LD_{10} of test drug (I.P). All mice were weighed and tumour sizes measured by caliper at weekly intervals. Tumour volumes were approximated as the product of the two longest perpendicular diameters. Every time the tumours were measured, control tumour volumes were compared with treated tumour volumes (Bellet et al, 1979).

1.6.3 Sub-renal Capsule Assay (SRCA): This assay involves insertion of small fragments (approximately $1mm^3$) of human tumour xenograft under the renal capsule of mice where there is a rich vascular bed ensuring both adequate nutrients for tumour growth and ready drug delivery. One of two types of assessment is used. in the first method, a stereoscopic microscope in which a micrometer disc is inserted into one eye piece is used. It is reportedly possible to measure in situ the size of the initial graft and the ultimate size achieved at the termination of the experiment. An assay time period of eleven days was selected since it was long enough to permit measurement of extent of growth and of drug-induced inhibition of the human tumour xenografts. In the second method, on days 1, 2 and 3 following tumour implantation, the mice were treated either with a placebo (control) or with various anticancer agents. Between days four and six, mice were sacrificed and the mean tumour diameter measured. The tumour bearing kidney is then fixed in bouins solution and processed for histological analysis after staining with haematoxylin-eosin. Seven histological parameters were blindly rated in a semi quantitative fashion using values between -3 to +11.
The criteria for drug activity against human tumour xenograft implanted subcutaneously and under the renal capsule are usually those set by the division of cancer treatment, National Cancer Institute, USA

(Goldin et al, 1981). These are 58% inhibition relative to controls for the S.C model and 80% inhibition for the subrenal capsule model. In addition, it is possible to use tumour weight as an endpoint. Tumour weight (W) in mg is estimated from caliper measurement according to the formula : $W = \frac{a^2}{2} \times b$

where **a** is the width and **b** is the length in mm.

In an effort to standardize the variability in tumour size among test groups at the initiation of treatment, Goldin et al (1981), calculated relative weight (RW) using the formula $RW = Wi/W_o$ where W_o is the mean tumour weight of a group at the beginning of treatment and Wi is the mean tumour weight at any subsequent time. A significant response to treatment is indicated when a test group shows an RW $\leq$ 42% of that of the control at any time during a specified range of days after the last treatment. The tumour xenograft models (both s-c and subrenal) may be more valuable than the L1210 and P388 leukaemia models as screening assays for new chemotherapeutic agents. There were fewer false positive results obtained using this assay as compared to murine L1210 or P388 models which make them potentially good secondary screens (Goldin et al, 1981).

1.7 Aims and Objectives of this Study:

Most of the common murine tumour models employed in the preclinical screening of antitumour drugs are lymphoid in origin (e.g L1210 and P388 leukaemias). While these models may be adequate for predicting response of human lymphoid leukaemias, they may not be the logical screens for drugs effective against myeloid leukaemias. One aim of the present study was to evaluate the responses of two

antileukaemic drugs (Ara-C and mitoxantrone) using murine myeloid leukaemias. The latter were induced in CBA/H mice following whole-body x-irradiation. Although both drugs are already in clinical use, their re-evaluation in the laboratory would provide information regarding the suitability of these murine myeloid leukaemias as preclinical screening models. Cytarabine is an established drug of choice for treating acute myeloid leukaemia and has been in clinical use for over twenty years. In contrast, mitoxantrone was recently introduced in the treatment of relapsed adult acute myeloid leukaemia. It would therefore be interesting to determine the similarities and differences between the two drugs bearing in mind that they belong to two distinct chemical classes. A variety of techniques such as dye-exclusion assay, [3H]thymidine uptake assay, autoradiography and granulocyte-macrophage colony forming cell (GM-CFC) assay would be employed to determine drug-induced cell lethality.

Myelosuppression is the dose-limiting toxicity of many antileukaemic drugs including cytarabine and mitoxantrone. The effects of both drugs on normal (murine) bone marrow cells would be monitored and compared to their effects on myeloid leukaemic cells. This will give an indication of any selective toxicity to leukaemic cells as compared to normal bone marrow cells.

Finally, an attempt would be made to study the in vivo activity of mitoxantrone against leukaemia bearing mice.

2. MATERIALS AND METHODS

CHAPTER TWO

2. MATERIALS AND METHODS

2.1.1 Mice and Housing: In all experiments, CBA/H mice inbred in the departmental animal house and exposed to 12-hour cycle (each) of light and darkness were used. They were fed on animal house feed RM1(C) 3/8 and were allowed chlorinated water ad libitum. Not more than 6 mice were housed per cage.

2.1.2 Leukaemias: The leukaemia models employed were passaged cell lines derived from transplantation of primary (1°) leukaemia induced following 3 Gy whole body X-irradiation in male mice aged 100 ± 10 days (Hepburn et al, 1987). The cell lines were given the code 81287 (1-10), SA2 (30-50), SA7 (100-180) and SA8 (90-150). The numbers in bracket indicate the range of passage number within which all experiments in that particular cell line were performed. For example, experiments using the 81287 cell line were performed between the 1st and 10th passage etc. Two pseudoprimary cell lines (given the code 10.12 and 10.13) were also used. The pseudoprimary leukaemias were derived as a result of transplanting bone marrow cells from mice irradiated with 3 Gy into mice that were irradiated with 4.5 Gy x-irradiation.

2.1.3 Autopsy Procedure: Overtly leukaemic mice which showed characteristic signs of pale feet (due to anaemia), ruffled fur and weight loss were killed by ether anaesthesia. In all the cell lines, there was leukaemic infiltration of spleen and therefore, the presence of splenomegaly together with examination of blood smears confirms

diagnosis of leukaemia. The femora were dissected free of muscle and fat and the marrow cells aspirated into RPMI medium supplemented with penicillin (50 I.U/ml), streptomycin sulphate (50ug/ml), 10% foetal calf serum (FCS) and 2mM glutamine using a No. 23 gauge needle using sterile technique. A single cell suspension was prepared by gentle syringing through No. 23 followed by No. 25 gauge needles. Spleen cells were similarly treated except that they were initially aspirated through a 1ml syringe without a needle. The upper portion of medium and cells were removed and a single cell suspension prepared as for the bone marrow.

2.1.4 Transplantation Procedure: To passage the leukaemias, spleen single cell suspensions in Fischers medium supplemented with glutamine and antibiotics were prepared as described above. Two morphologically distinct cell lines were derived depending on the number of cells routinely passaged (Hepburn et al, 1987). When 1×10^6 cells were routinely injected intraperitoneally (I.P), the mice became overtly leukaemic 7 days later. This is termed "high cell dose passage"(HD). In contrast, when 1×10^4 cells were routinely injected intravenously (I.V), the recipient mice became moribund with leukaemia 30 days after tumour inoculation. This is called "low cell dose passage"(LD).

2.1.5 Drugs: Cytosine arabinoside (1,ß-D-Arabinofuranosyl cytosine; cytarabine; Ara-C) was purchased from Sigma Chemical Company, St Louis, (USA) as the free base and was dissolved in RPMI or Dulbeccos medium depending on the type of experiment.

Mitoxantrone hydrochloride (Mitozantrone, Novantrone(R), Mitox) 2mg/ml, was a generous gift from Lederle Laboratories, Hampshire,

England. Serial dilutions of the drugs were made in appropriate media (or saline) just before setting up the experiments.

2. 2 CELL CULTURE ASSAYS

2.2.1 Differential Staining Cytotoxicity (DiSC) Assay: This assay is based on differential staining of live and dead cells so that they are easily distinguishable from each other.

2.2.1.1 Stains: Fastgreen (FCF No F252) and nigrosin (N-4754) were purchased from Sigma Chemical Company, St Louis, (U S A). Harris haematoxylin was prepared as follows:

Haematoxylin : 1g

Absolute alcohol : 10ml

Ammonium or potassium alum : 2g

Mercuric oxide : 0.5g

The haematoxylin was dissolved in alcohol and alum (previously dissolved in hot water) added. The mixture was quickly boiled and mercuric oxide added when the solution turn purple. It was then rapidly cooled under tap water and filtered before use. Acetic acid (8 ml) was added to sharpen nuclear staining.

2.2.1.2 Internal Standard of Pigeon Red Cells: Blood (10ml) was obtained from a pigeon in a bottle containing an anticoagulant. The pigeon red cells (PRC) were washed three times with 0.15M NaCl. They were then resuspended in 2% acetaldehyde in phosphate buffered saline by dropwise addition of packed red cells into the acetaldehyde with continuous stirring on a magnetic stirring plate. Cells were left in this buffer for ten days and were then sterilized by soaking in 99% ethanol overnight followed by extensive washing with sterile 0.15M NaCl. The cells were finally suspended in 0.15M NaCl containing streptomycin (100

ug/ml) and penicillin (100 i.u/ml). The cells were adjusted to haematocrit of 2.5% (volume of PRC over total volume of PRC plus suspending medium). This working suspension was stored at 4°C and was of sufficient quantity for many assays. Prior to use in the assay, the working suspension was diluted 1:40 to approximately 7×10^6 PRC/ml. One half of this suspension was added to 20ml fastgreen-nigrosin. The pigeon red cells were easily identifiable as nucleated microelliptocytes (Weisenthal et al, 1983 b).

2.2.1.3 Preparation of Slides: 100µl of cells (7×10^5 cells/ml in RPMI medium supplemented with FCS and antibiotics) were plated in a microtitre plate (Nunclon). 100ul WEHi conditioned medium was added to give a final concentration of 10%. This is the optimal concentration for cell proliferation stimulation. 100 ul each of Ara-C, mitoxantrone or combination of the two, or medium (control) was added per well and left in culture for the entire period of the assay. After 4 days incubation at 37°C in a humidified atmosphere containing 5% CO_2 in air, six wells were pooled and mixed 1:1 (V/V) with dye solution containing fast-green nigrosin to which permanently fixed pigeon red cells were added. The mixture was briefly mixed and after 10 minutes at room temperature it was cytocentrifuged onto ethanol washed slides and air dried.

2.2.1.4 Haematoxylin-Eosin Staining: Eosin was prepared as follows:

1% Eosin - 100ml

1% Phloxin - 10ml

95% ethanol - 780ml

Glacial acetic acid: 4ml

Slides fresh from cytocentrifuge were stained (without prefixing) in Harris formula haematoxylin. After 90 seconds, the staining rack was

removed and transferred to a solution of 7.5% ethanol for two quick dips lasting less than five seconds (sec). Following this, the rack was quickly dipped twice in additional changes of 7.5% ethanol to remove excess haematoxylin before eosin staining. Following the ethanol washes, the slides were placed in the eosin solution for 30 sec during which time fixation also took place. The slides were then briefly dehydrated by 2 successive quick dips in 95% ethanol, 100% ethanol and xylene. The slides were then allowed to dry and mounted. Living cells were stained pink with haematoxylin-eosin and dead cells were stained dark-green with fast green-nigrosin. Alternatively slides were counterstained with Diff-Quik (Merz and Dade, AG, Switzerland) or Jenner- Giemsa which not only gives comparable results but was also less tedious (Bird, Bosanquet and Gilby, 1985).

2.2.1. 5 Diff- Quik^{R} Staining : Cytospin slides were prepared and allowed to dry in the air. They were stained in the following manner:

1. Slides were dipped 6 times in fixative solution (fastgreen in methanol (0.002g/L)) with each dip lasting not more than 5 seconds. The excess fixative was allowed to drain after each dip.
2. The slides were dipped 6 times in solution 1 (eosin G in phosphate buffer at pH 6.6) with each dip lasting not more than 5 seconds.
3. The slides were dipped 6 times in solution 2 (thiazine dye in phosphate buffer at pH 6.6) with each dip lasting not more than 5 seconds.
4. The slides were rinsed with water and allowed to dry. They were then mounted in euparol.

2.2.1.6 Jenner - Giemsa Staining: Cytospins were prepared and dried in air and were then fixed by immersing them in a jar of methanol for 10 -

20 minutes. They were then transferred to a staining jar containing Jenner stain freshly diluted with an equal volume of water. After staining for 5 minutes the slides were immersed (without washing) in Giemsa stain (freshly diluted with nine volumes of water). After staining for 10 minutes the slides were transferred in to a jar containing tap water and allowed to stand for 2 - 5 minutes. The slides were stood upright to dry. They were mounted in euparol.

2.2.1.7 Assay Interpretation: For the determinations of drug-induced cytotoxicity the ratio of live tumour cells over simultaneously counted pigeon red cells was determined for each slide and the ratio in drug-treated samples expressed as percentage of that in the control. This expression was termed "tumour cell viability" (TCV). The addition of PRC overcomes the problems of cell lysis and proliferation in culture thus allowing direct comparison of the number of surviving cells present in control and drug treated samples (Bird et al, 1986).

2.2.2 [3H]-Thymidine Uptake Assay: The tritiated thymidine ([3H]TdR) uptake assay measures the ability of proliferating cells to take up [3H]-thymidine for incorporation into DNA during DNA synthesis (S-Phase). The degree of DNA synthesis Inhibition in drug treated samples compared to the untreated control gives a measure of cytotoxicity produced by a drug.

Leukaemic or normal bone marrow single cell suspensions were prepared as described previously (section 2.1.3). 100µl cells (7×10^5 cells/ml) in RPMI medium supplemented with foetal calf serum (10%), penicillin 100 i.u/ml), streptomycin (50µg/ml) and glutamine (2mM) were plated in microtitre plates (Nunclon) together with 100µl WEHi conditioned medium (to give a final concentration of 10%) and then

100µl drug or medium (control) was added. In the continuous incubation method, the drugs were left in culture for the duration of the experiment. For the 1 hour pulse method, cells were incubated with one drug or medium (control) for one hour, washed twice and resuspended in RPMI medium and plated out after which the second drug was added either immediately or 24, 48, or 72 hours later. After four days incubation in humidified atmosphere containing 5% CO_2 , 7.5KBq [3H]TdR (Specific activity 777MBq/mg) (Amersham International Plc, Buckinghamshire, England) was added per well and the plate further incubated for eight hours. The cells were harvested onto a glass fibre filter paper (Titertek, Flow Laboratories, Irvine, Scotland) using a cell harvester (Titertek, Skatron, Norway). After at least 12 hours drying in an oven, individual filter discs were placed in scintillation vials and 2ml of scintillant (Optiphase 'Safe', FSA Laboratory, Loughborough, England) added and radioactivity deposited on the discs determined using a liquid scintillation counter (LKB 1214 Rackbeta). Dose-response curves were obtained by plotting counts per minute in drug treated samples as a percentage of untreated control uptakes as a function of the drug concentration.

Definations of Additivity, Synergy and Antagonism: An additive effect is manifested when the combined effect of two drugs acting either by the same mechanism (Levine, 1983) or via different mechanisms (Grundy, 1985) is equal to that expected by simple addition. When the joint effect of two drugs is greater than the algebraic sum of their individual effects a synergistic effect is said to be manifested (Levine, 1983). An antagonistic effect is manifested when the effect produced by a combination of two drugs is less than the sum of the effects produced by the single drugs (Levine, 1983).

2.2.3 Cell Counting using Coulter Counter: Microtitre plates were set up as described under Section 2.2.2. On day 4, following drug incubation, six wells were pooled (each) from control and drug treated wells into coulter vial containing isoton (20ml). Three drops of Zaponin (Coulter Electronics, England) were added and the counts determined using a coulter counter (model Zm, Coulter Electronics, England) using the following settings:

current - 130

full-scale - 10

lower threshold - 30

Attenuation - 1

2.2.4 Autoradiography: This technique is usually employed to determine the number of cells that are actively proliferating in a cell population. The sample is labelled with radioactive DNA precursor which is usually [3H]- thymidine. A thin layer of photographic emulsion is placed on sections of the sample to be studied and left in the dark for a period of days. When the photographic emulsion is developed, those cells which contained radioactively labelled DNA in their nuclei will have black silver grains deposited in the emulsion above them. The grains are caused by radioactive emission. Because ß-particles emitted by tritium have less energy and therefore shorter range, the silver grains are clustered neatly over the labelled nuclei (Aherne, Cample-John and Wright, 1977).

2.2.4.1 Preparation of Slides: Plates were set up as for microtitre assay described previously (section 2.2.2). On day 4 following incubation in humidified atmosphere containing 5% CO_2 in air, 7.5KBq 3HTdR was added per well and the plates further incubated for one hour. At least six

wells were pooled, washed twice and cells resuspended in RPMI medium (supplemented with serum to ensure good cytology). Cytospins were prepared onto ethanol washed slides using a Shandon 2-cytospin at 800 rpm for ten minutes.

2.2.4.2 Autoradiographic Process: Each slide was dipped in K_2 liquid emulsion in gel form (Illford nuclear research emulsion) which has been diluted (1:1) with 1% glycerol solution. After drying, slides were boxed and kept in the dark at 4°C for four days. They were then developed. This involved dipping the slides in D19 high contrast developer (Kodak 5027065) for 3.5 minutes followed by rinsing for 30 seconds in distilled water and fixing for 5 minutes in Kodak acid fixer after which they were rinsed in running water for 5 minutes. After drying, the slides were stained with Jenner - Giemsa, the Jenner being previously filtered to remove any precipitation. The slides were allowed to dry and mounted in euparol.

2.2.4.3 Labelling Index: All slides were scored in a blind fashion under oil immersion (x40). At least 400 cells were counted per slide unless cellularity was adversely reduced by drug treatment. The background grain count was determined from acellular field on the slide and the appropriate threshold value for a cell to be classified as labelled decided from this. The labelling index was taken as the fraction of labelled cells expressed as a percentage of the whole cell population.

2.2.5 Granulocyte-Macrophage Colony Forming Cell Assay: The cytotoxic effects of drugs on normal myeloid progenitor cells were determined using granulocyte-macrophage colony forming cell (GM-CFC) assay. Cells were either pulsed with drugs for one hour, washed

twice and then plated in agar or else the drugs were dissolved in the same medium in which the cells were suspended (i.e continuous exposure).

To 8ml of Dulbecco's medium supplemented with horse serum (20%), glutamine and antibiotics, was added 1ml of agar (3%) and 1ml of cell suspension ($5x10^5$ cells/ml). The mixture was briefly shaken and 1ml aliquoted into 30mm petridish containing 0.1ml WEHi conditioned medium as a source of colony stimulating activity. The plates were incubated in a humidified atmosphere containing 5% CO_2 in air at 37°C.

2.2.5.1. Assay Interpretation: Colonies (> 50 cells) were scored using an inverted microscope on day 7. Dose-response curves were obtained by plotting the number of colonies in drug treated samples as a percentage of control colony numbers against the concentrations of the drugs used. At least 40 colonies must be present in control plates for an experiment to be considered evaluable.

2.3.1 In Vivo Chemotherapeutic Study

Female CBA/H mice weighing 20-25g and 17 weeks old were inoculated with leukaemic cells. On days 1 and 5 following tumour inoculation, mitoxantrone (freshly diluted in normal saline) was administered. Control mice received only leukaemic cells and saline. The mice were observed daily and if they showed any or a combination of these symptoms, they were autopsied in order to determine the cause of death: weight loss, pale feet, conjunctivitis, ruffled fur, etc.

An increase in life-span of drug treated mice relative to controls was used as an end point for assessing drug activity. The survival of treated mice counted from day of tumour inoculation was determined and

compared to untreated control and the ratio of the two expressed as per cent increase in life- span (% ILS). The following formula was used to calculate (%)ILS (Fujimoto and Ogawa, 1982):

$$\frac{(T-1)}{C} \times 100$$

where:

T = mean number of days survived by treated mice

C = mean number of days survived by control (untreated) mice

2.3.2 Normal Mice: Normal mice of similar weight and sex as leukaemic mice received similar schedule of mitoxantrone. The mice were sacrificed and spleen weight, femur cellularity, haemoglobin count (Hb) and haematocrit (packed cell volume, PCV) determined as follows:

Femur Cellularity: Bone marrow cells were flushed from a femoral bone using 3mls of RPMI medium supplemented with serum (10% foetal calf); glutamine and antibiotics. A single cell suspension was then prepared (Section 2.1.3). The cell suspension(100µl) was added to isoton (20ml) in a vial and 3 drops of Zaponin (to lyse red cells) added. The number of nucleated cells per millilitre (ml) was then determined from the coulter counts. And the femur cellularity was obtained by multiplying this by 3.

Spleen Weight: Spleen was dissected free from fat and muscle and weighed.

Haemoglobin Count: Blood was collected following cardiac puncture in an anaesthetized mice and stored in heparinized tubes. The tubes were gently shaken in order to ensure proper mixing of blood and anticoagulant to prevent clotting. A haemoglobin photometer was

calibrated using standard microcuvettes. The blood sample in a microcuvette chamber was then inserted into the photometer and the haemoglobin count (g/L) read off.

Packed Cell Volume (PCV): Blood was drawn into a capillary tube one end of which was sealed with crystaseal. The tubes were cytocentrifuged using a microhaematocrit centrifuge. The packed cell volume was then read off using standard methods.

3. RESULTS

CHAPTER THREE

3. RESULTS

3. In vitro Chemotherapeutic Assays

This chapter contains the results from a series of experiments where the responses of myeloid leukaemic cells were investigated following treatment with cytotoxic drugs used singly or in combination. The responses were monitored using the following assays :-

cell viability assay using fast green containing fixed pigeon red cells

- the DiSC assay (section 3.1);

Assessment of cell number using coulter counter (section 3.1.3);

[3H]-thymidine uptake assay following growth factor stimulation of cell proliferation (section 3.2);

Autoradiography following labeling the cells with [3H]-thymidine [[3H]TdR] (section 3.3.);

Granulocyte-macrophage colony forming cell (GM-CFC) assay following treatment of normal bone marrow cells with drugs and plating the cells in agar (section 3.4).

3.1 Differential Staining Cytotoxicity (DiSC) Assay

Under this heading two assays were employed: pigeon red cell (Section 3.1.2) and assessment of cell number using coulter counter (Section 3.1.3).

Prior to setting up the pigeon red cell (DiSC) assay the methodology was evaluated by determining the ratio of live and dead cells in a mixed population (Section 3.1.1). The responses of the following leukaemic cell lines to Ara-C, mitoxantrone either singly or in combination were determined using both the DiSC assay and assessment of cell number using coulter counter: SA2, high cell dose (HD) transplant of SA7 (SA7HD) and SA8 high cell dose passage (SA8HD).

3.1.1 Evaluation of Pigeon Red Cell (DiSC) Method: Leukaemic bone marrow single cell suspension was prepared from femora of mice bearing the SA7HD leukaemia as described under section 2.1.3. Some of the cells were killed by heating at 60°C for one hour and mixed with live cells to give 0, 25, 50, 60, 80 and 100% live cells. Viability was determined after staining with fastgreen-nigrosin and counterstaining with Jenner-Giemsa.

Results: There was a good correlation between expected and observed values (Figure 1) and this indicated that the procedure does preserve and differentiate live and dead cells in a mixed population. Live cells assumed normal Jenner-Giemsa counterstain (pink) while dead cells were stained dark-green by fastgreen-nigrosin(Plate 1).

3.1.2 Pigeon Red Cell (DiSC) Method: A single cell suspension was prepared from the following leukaemic cell lines (SA2, SA7HD, SA8HD) and plated at 7 x 10^4 cells per well in a microtitre plate. WEHi conditioned medium was added (except cells from SA2 cell line) to give a final concentration of 10% per well. 100µl each of mitoxantrone, Ara-C or combinations of the two was added to give final concentrations in the range 0.12-120ng/ml. In combination experiments, the concentration of one drug was varied (0.12-120ng/ml) while maintaining the final concentration of the second drug at 1.2ng/ml. After four days incubation, six wells were pooled and the cells were stained with fastgreen-nigrosin to which permanently fixed pigeon red cells were added. They were then cytocentrifuged onto ethanol-washed slides. After Jenner-Giemsa counter staining, the ratio of live cells over simultaneously counted pigeon red cells was determined (blindly) for each slide and the ratio in drug-treated samples expressed as a percentage of that in control. This expression called tumour cell viability (TCV) was plotted against drug concentrations to yield dose-response curves.

3.1.3 Assessment of Cell Number: Experiments were set up as described under section 3.1.2. On day 4 following drug incubations, six wells were pooled (each) from drug treated and control samples and counted using a coulter particle counter (see Section 2.2.3). Dose-response curves were obtained by plotting the ratio of counts in drug treated samples as a percentage of that in control as function of drug concentrations.

Results: The responses of the leukaemic cell line SA2 to Ara-C alone and in combination with mitoxantrone (1.2ng/ml determined using both pigeon red cell (DISC) and coulter (CC) methods are shown in Figure 2. According to both methods, the cell line was not sensitive to Ara-C within the concentration range 0.12-12ng/ml as only slight

increase in cytotoxicity was observed with increasing dose within that range. However, a marked cytotoxic effect was seen with 120ng/ml in the DiSC assay. There was a close agreement between the two methods although the electronic particle count method seemed to underestimate

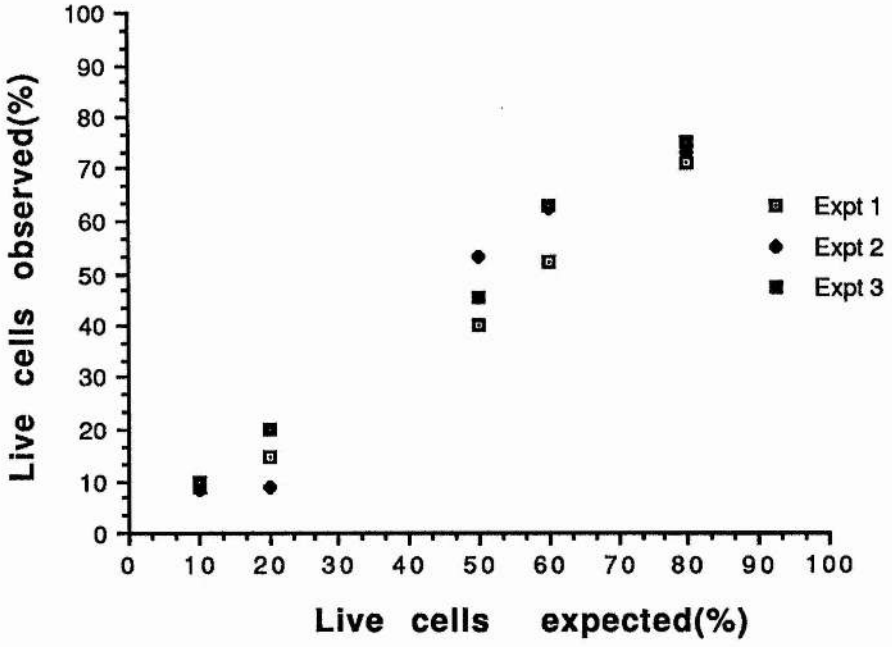


Fig.1 : Percentage of live cells observed after staining known proportions of live and dead cells with fast green.

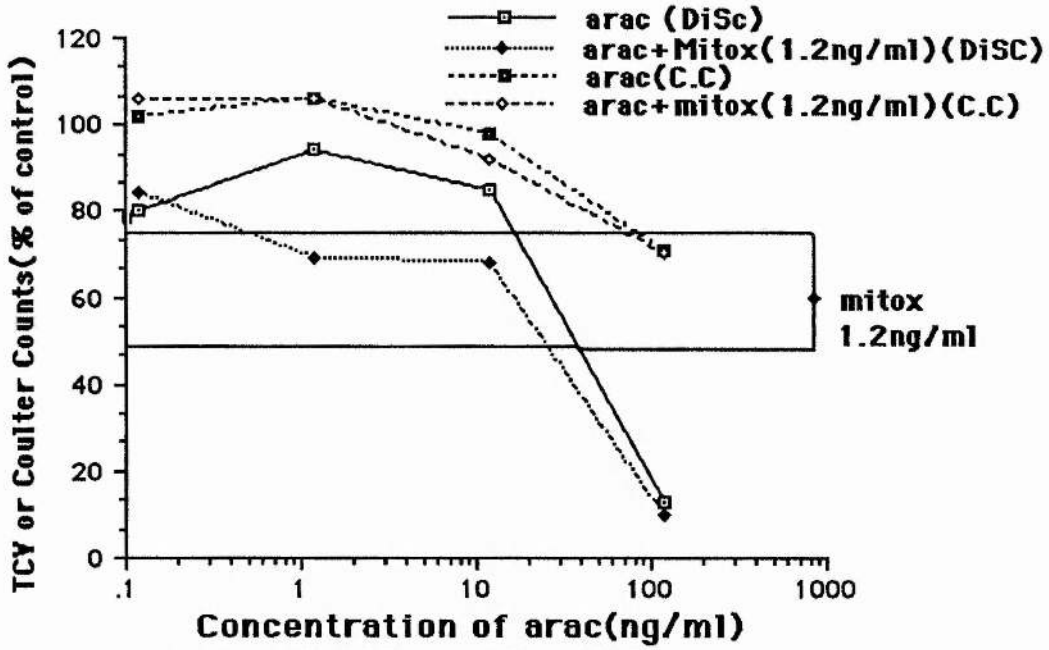


Fig.2: The response of SA2 leukaemic cell line to arac alone or in combination with mitoxantrone assesed using the DiSC assay and coulter count method.

cytotoxicity produced using the highest concentration employed (120ng/ml). Whilst a slight additive effect was observed when mitoxantrone (1.2ng/ml) was added to Ara-C (in the concentration range 1.2-12ng/ml) in the DiSC assay, no such effect was observed using the coulter method. In the latter case where mitoxantrone was added to Ara-C, there was no difference from the response with Ara-C alone. Figure 3 shows the effect of mitoxantrone alone or in combination with Ara-C (1.2ng/ml) on the same (SA2) leukaemic cell line using both methods. Again there was close agreement between the dose response curves obtained using the two methods. As was observed with Ara-C, mitoxantrone within the concentration range 0.12-12ng/ml had virtually no effect on cytotoxicity. However, increased cytotoxicity was observed when higher concentration of mitoxantrone was used (120ng/ml). Mitoxantrone alone was no better than Ara-C alone in the extent of cell kill produced. Addition of Ara-C to mitoxantrone resulted in either no

effect (cell count) or antagonism at low concentrations of mitoxantrone (DiSC).

The responses of the SA7 cell line to Ara-C alone and in combination with mitoxantrone are shown in Figure 4. This cell line seemed slightly more sensitive to Ara-C alone as compared to the SA2 cell line using the cell number assessment method. There was a close agreement between the two methods in determining the response of the cell line to Ara-C alone. Addition of mitoxantrone (1.2ng/ml) to Ara-C resulted in either no increased effect (DiSC) or an additive effect (CC). This cell line seemed more sensitive than SA2 to mitoxantrone alone and in combination with Ara-C (Figure 5) as assessed by both DiSC and cell number assessment methods. Addition of Ara-C to mitoxantrone resulted in antagonism at the lowest mitoxantrone concentration (0.12ng/ml)(DiSC) or slight additive effect (CC). Figure 6 shows the dose-response curve of the SA8 cell line to Ara-C alone and in combination with mitoxantrone. At low to intermediate concentrations (0.12-12ng/ml), Ara-C had little effect on cytotoxicity as assessed by both methods. When the dose of Ara-C was increased to 120ng/ml there was about an 80% increase in cytotoxicity relative to control. Both methods gave almost identical dose-response curves. Addition of mitoxantrone to Ara-C, resulted in additive cytotoxic effects as determined using both methods. The SA8 cell line is as sensitive as the SA7 cell line to mitoxantrone (CC) (Figure 7). Unlike what was observed with Ara-C, increasing the concentration of mitoxantrone within the range 0.12-12ng/ml was associated with increased cytotoxicity.

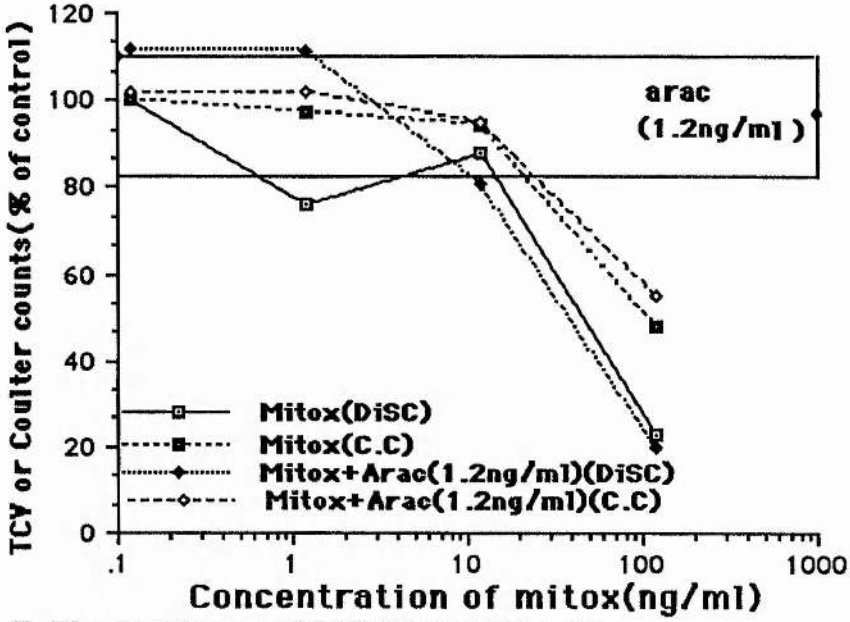


Fig.3: The responses of SA2 leukaemic cell line to mitox alone or in combination with arac assessed using the DiSC assay and the coulter count method.

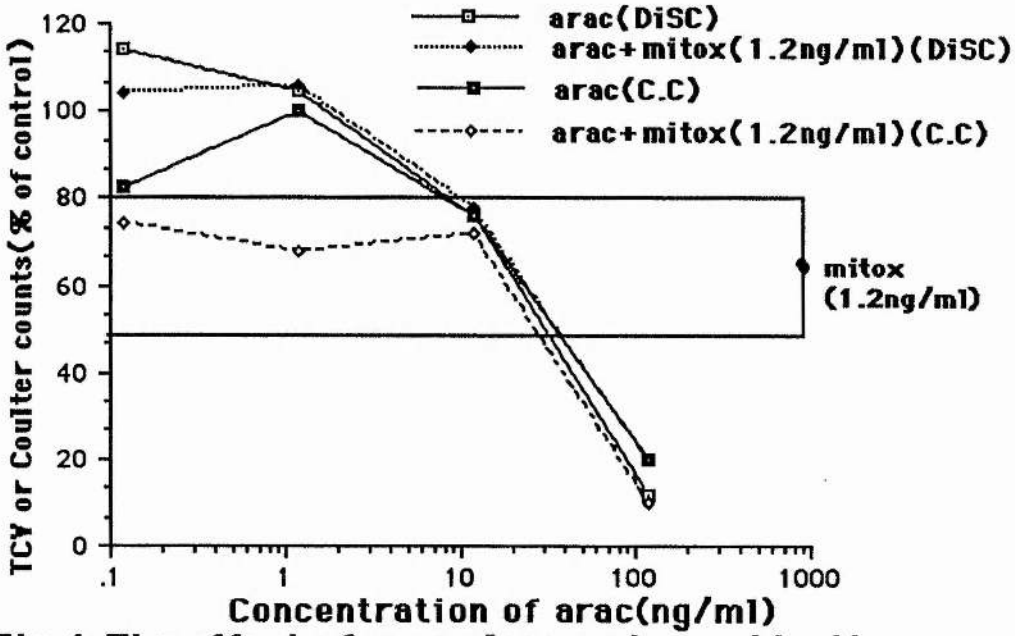


Fig.4: The effect of arac alone or in combination with mitox on SA7 leukaemic cell line monitored using the DiSC assay and the coulter count method.

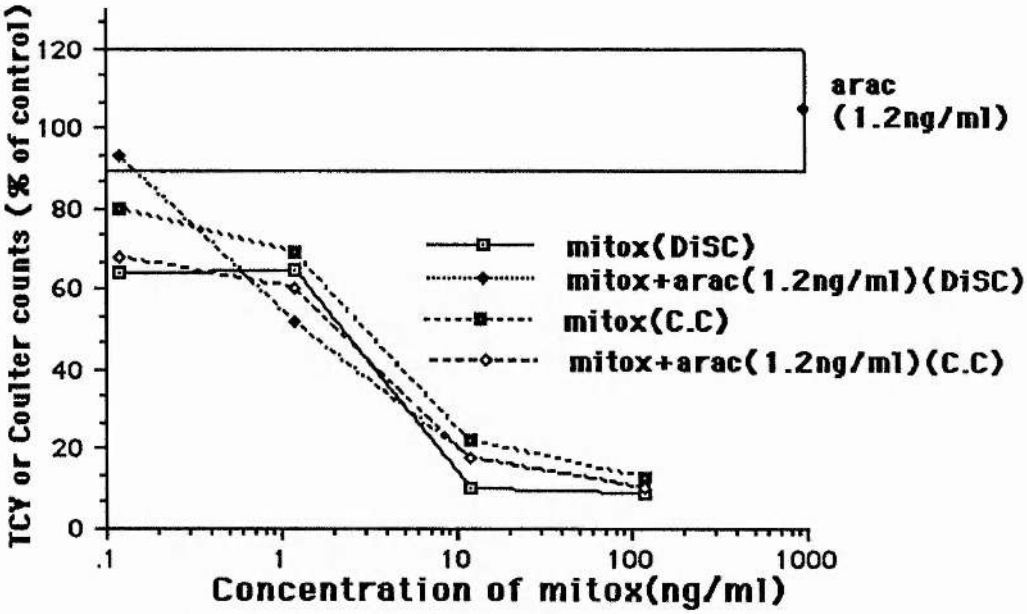


Fig.5: The effect of mitox alone or in combination with arac on the SA7 leukaemic cell line monitored using the DiSC assay and the coulter count method.

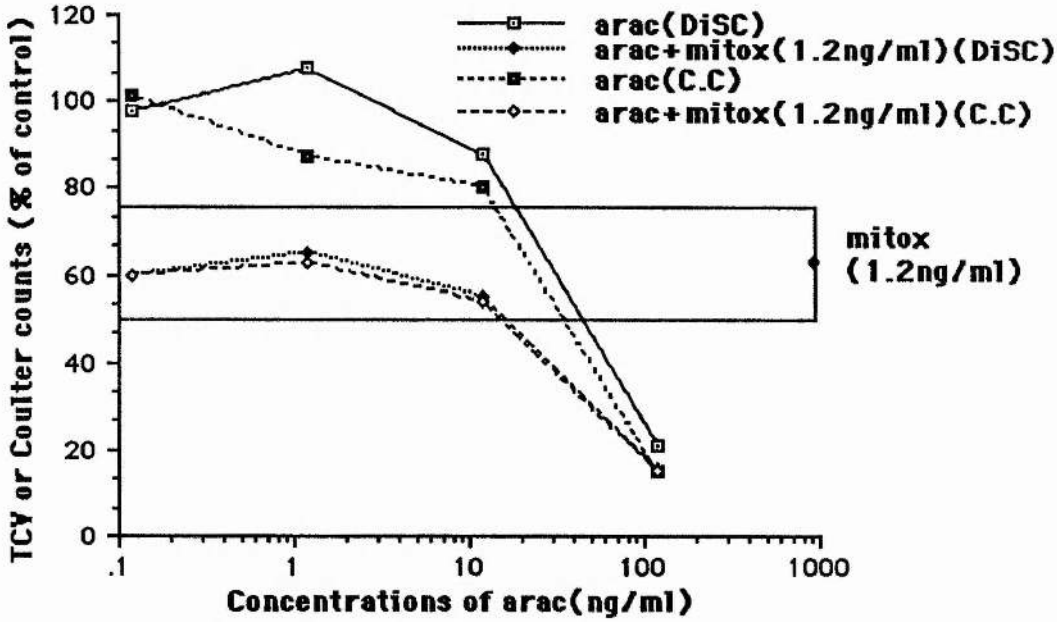


Fig.6: Cytotoxic effects of arac alone or in combination with mitox on SA8 leukaemic cell line monitored using the DiSC assay and the coulter counter method.

However, only the DiSC assay showed an additive effect when Ara-C (1.2ng/ml) was added to mitoxantrone. With the cell number assessment method, addition of Ara-C did not result in increased cytotoxicity as compared to mitoxantrone alone.

There was no difference in potency between mitoxantrone and Ara-C on their effect on SA2 cell line. However, mitoxantrone seem more potent than Ara-C at the 12ng/ml concentration against both SA7HD and SA8HD cell lines.

3.2 Tritiated Thymidine Uptake Assay: Using this assay, the effects of Ara-C, mitoxantrone or their combinations on leukaemic cell lines 81287; 10.12; 10.13; SA2; SA7 low cell dose passage (SA7LD); SA7 high cell dose passage (SA7HD); SA8 low cell dose passage (SA8LD); SA8 high cell dose passage (SA8HD) and normal bone marrow (NBM) cells were determined. Prior to setting up drug experiments, the amount of growth factor required for optimum in vitro cell proliferation stimulation was determined (section 3.2.1). An experiment was also set up to determine the effect of foetal calf serum on in vitro mitoxantrone cytotoxicity (section 3.2.2).

Drug effects were monitored either following continuous incubation (section 3.2.3) or a one hour pulse (section 3.2.4). In the continuous incubation method, the drugs were left in culture for the duration of the assay. In contrast, with the one hour pulse method, the cells were pulsed with drug(s) for one hour, followed by washing twice and a second drug was then added either immediately or hours later. In both methods, the assays were terminated on day 4 by addition of [3H]TdR followed by determination of the amount of radioactivity incorporated by drug treated and control samples.

3.2.1 Growth Factor Dose-Response Curve: Microtitre plates were set up as described under section 2.2.2. Graded concentrations of WEHi-conditioned medium (100µl) was added to give final concentration of 5, 10 or 20% in each well. After 4 days incubation, cells were labelled with [3H]TdR and harvested. The amount of radioactivity incorporated was determined using a scintillation counter.

Results: Figure 8 shows a typical growth factor dose-response curve obtained by incubating normal bone marrow cells with various concentrations of WEHi conditioned medium. It is apparent from the curve that 10% WEHi produced optimum effect on cell proliferation. Therefore, this concentration of growth factor was routinely used to stimulate cell proliferation in all in vitro assays reported here except those involving the SA2 leukaemic cell line.

3.2.2 Effect of Foetal Calf Serum on Mitoxantrone Cytotoxicity In Vitro: The aim of this experiment was to determine whether the presence of serum affects mitoxantrone-induced cell lethality in vitro. 7×10^4 normal bone marrow cells were plated per well in a microtitre plate and WEHi conditioned medium was added to give a final concentration of 10%. 100µl of mitoxantrone dissolved in either serum free medium or in medium containing 10% foetal calf serum was added to give final concentrations in the range of 0.12-120ng/ml. After 4 days incubation the wells were labelled, harvested, and the amount of radioactivity incorporated by control and drug treated cells determined. Two dose-response curves were plotted : one each for mitoxantrone dissolved in either serum free medium or in serum containing medium.

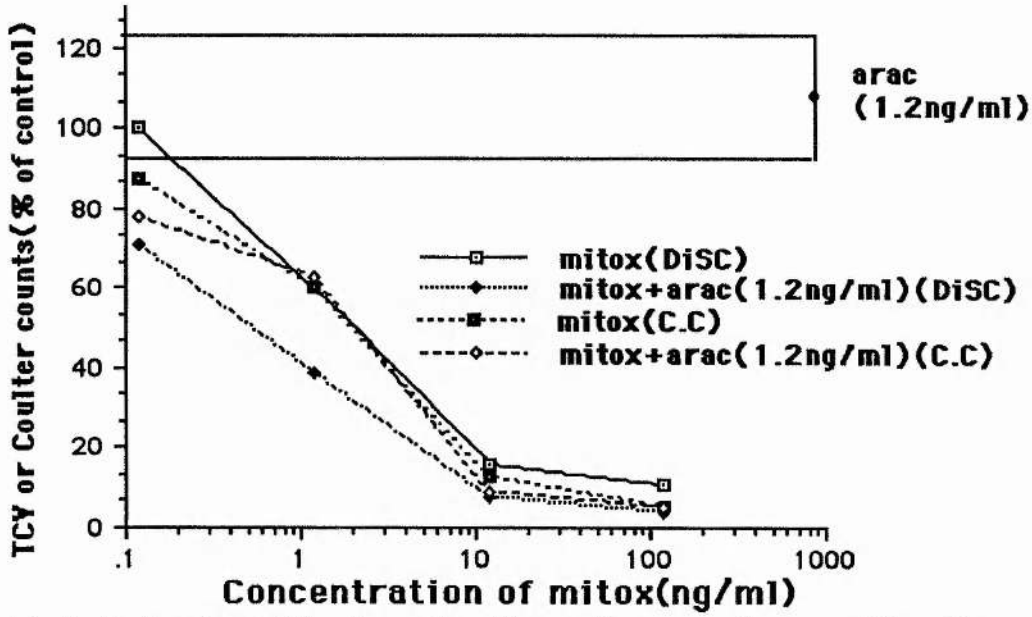


Fig.7: Cytotoxic effects of mitox alone or in combination with arac on SA8 leukaemic cell line monitored using the DiSC assay and the coulter count method.

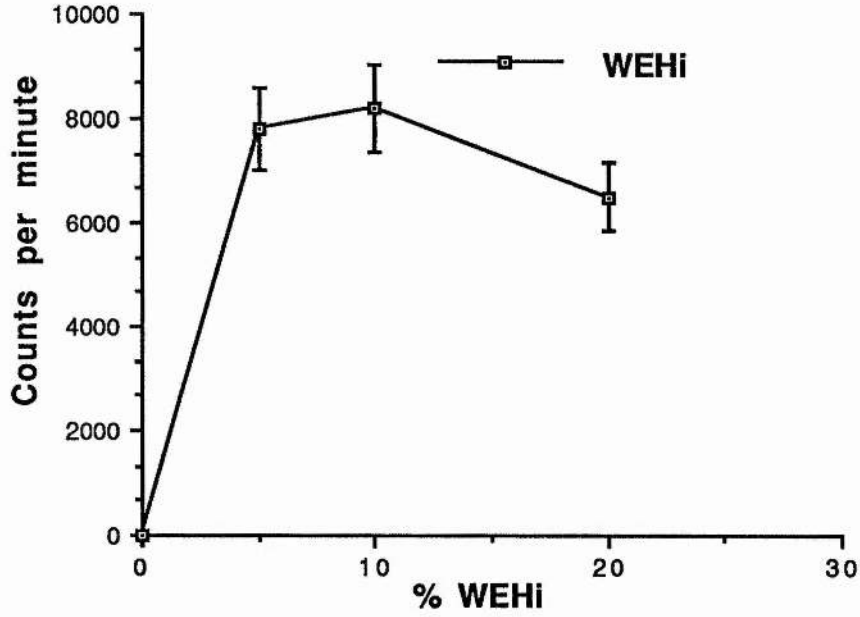


Fig.8: The response of normal bone marrow (NBM) cells to WEHi conditioned medium monitored using (3H)thymidine uptake assay.

Results: The presence of serum had no effect on mitoxantrone cytotoxicity in vitro (Figure 9). In contrast, there was a slightly increased inhibition of [3H]TdR uptake when mitoxantrone was dissolved in medium containing serum as compared to serum-free medium.

3.2.3 Continuous Incubation: In these experiments, leukaemic cells from the cell lines 81287, 10.12, 10.13, SA2, SA7HD, SA7LD; SA8LD, SA8HD and normal (murine) bone marrow cells were incubated with Ara-C, mitoxantrone or combinations of the two drugs. The drugs were left in culture for the duration of the experiment (four days). For the primary (81287) and low cell dose transplant cell lines, spleen cells were set up in addition to bone marrow cells.

A single cell suspension was prepared from the following leukaemic cell lines (81287, 10.12, 10.13, SA2, SA7, SA8) and NBM cells and plated at 7×10^4 cells per well. 100µl WEHi conditioned medium was then added (except experiments with SA2 cell line) followed by 100µl of drug (Ara-C or mitoxantrone) to give final concentration per well in the range 0.12-120ng/ml. In the combination experiments, the concentration of one of the two drugs was varied (0.12-120ng/ml) while maintaining the concentration of the second drug at 1.2ng/ml (unless otherwise stated). Dose-response curves were obtained by plotting counts per minute in the drug treated sample as a percentage of control uptake as a function of dose.

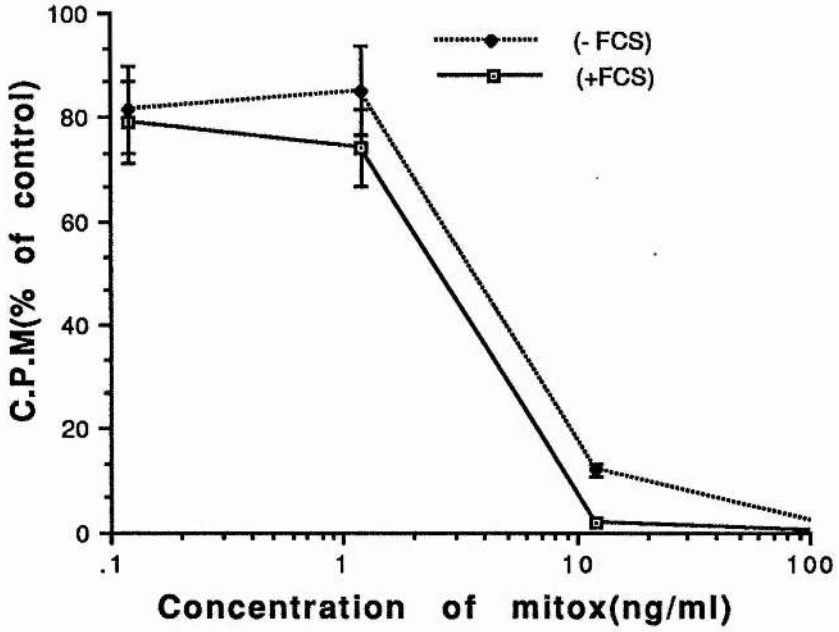


Fig.9: The efffect of foetal calf serum(FCS) on the cytotoxicity of mitox against NBM cells monitored using(3H)-thymidine uptake assay.

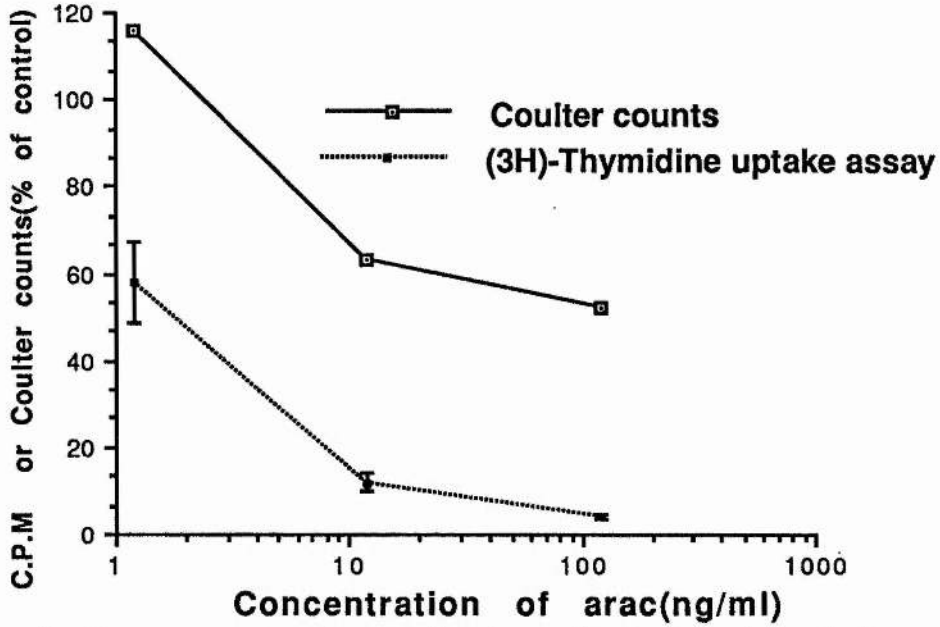


Fig.10: Response of spleen cells from the leukaemic cell line 81287 to arac determined using coulter counts and (3H)-thymidine uptake assay.

Results:

Primary (81287) and Pseudoprimaries (10.12 and 10.13) Leukaemic Cell Lines: Figure 10 shows the response of spleen cells from a Primary leukaemic cell line 81287 to Ara-C determined using [3H]TdR uptake assay. Similar dose-response curve was obtained by the cell number assessment method although cytotoxicity seem less pronounced according to the latter method. The response of the same spleen cells to mitoxantrone is shown in Figure 11. Again, there was qualitative similarity between the two dose-response curves although cytotoxicity was less pronounced using the electronic cell number assessment method even at the highest mitoxantrone concentration used (120ng/ml). Spleen cells seem more sensitive to mitoxantrone as compared to Ara-C according to the [3H]TdR uptake inhibition results. After the leukaemic cells were passaged in syngeneic mice (81287TI), spleen cells showed a slight decrease in sensitivity to Ara-C (Figure 12) and mitoxantrone (Figure 13). A comparison of sensitivity of spleen cells and bone marrow cells (Figure 14) to Ara-C suggests that spleen cells were slightly more sensitive at low concentrations. Similarly, spleen cells (Figure 13) were more sensitive to mitoxantrone as compared to bone marrow cells (Figure 15) at low concentrations. By the sixth passage sensitivity to Ara-C was decreased particularly at 12ng/ml (Figure 16). Below this concentration of Ara-C, virtually no effect was produced. Addition of mitoxantrone (0.12ng/ml) resulted in a slight additive effect at low concentrations which was to be expected since mitoxantrone alone at that concentration produced virtually little effect (Figure 17).

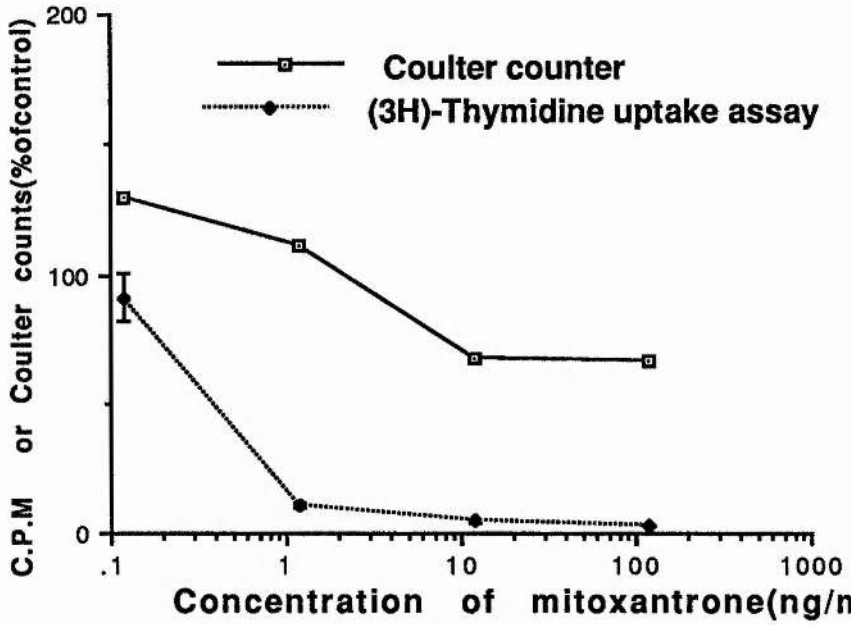


Fig.11: Response of spleen cells from primary leukaemic cell line 81287 to mitoxantrone monitored using coulter counter and (3H)-thymidine uptake assay.

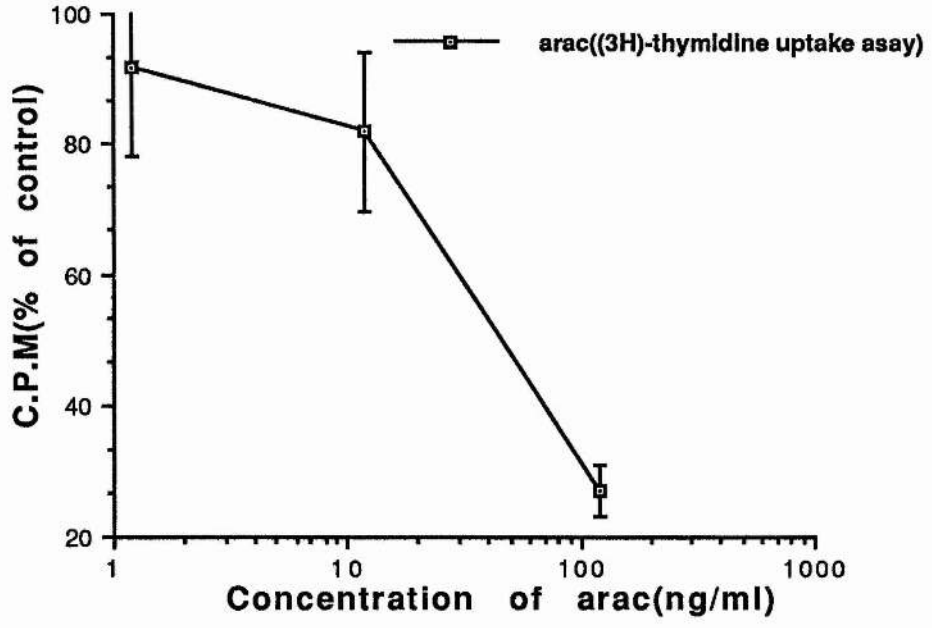


Fig.12: Response of spleen cells from 81287 cell line to arac monitored using (3H)-thymidine uptake assay.

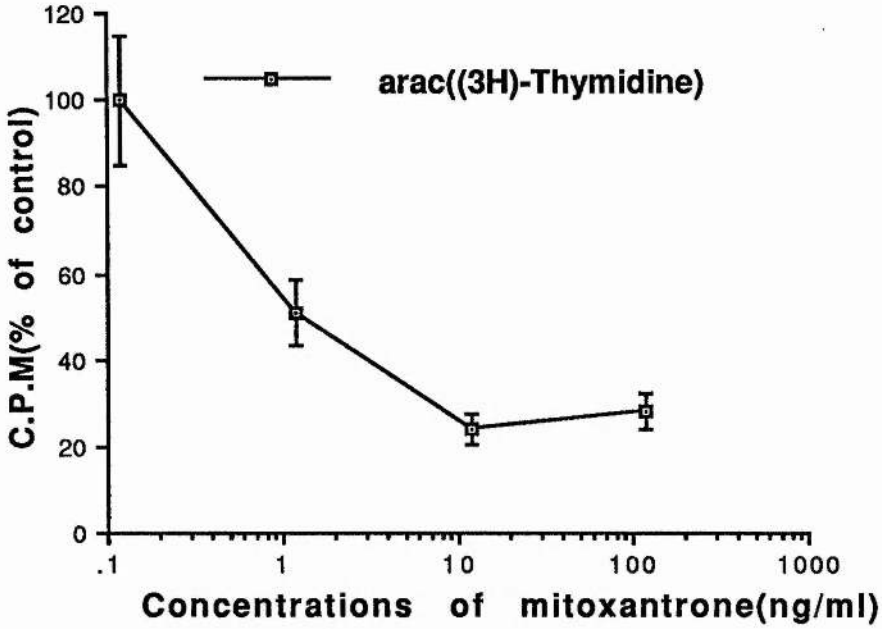


Fig.13: Response of spleen cells from 81287T1 to arac monitored using (3H) thymidine uptake assay.

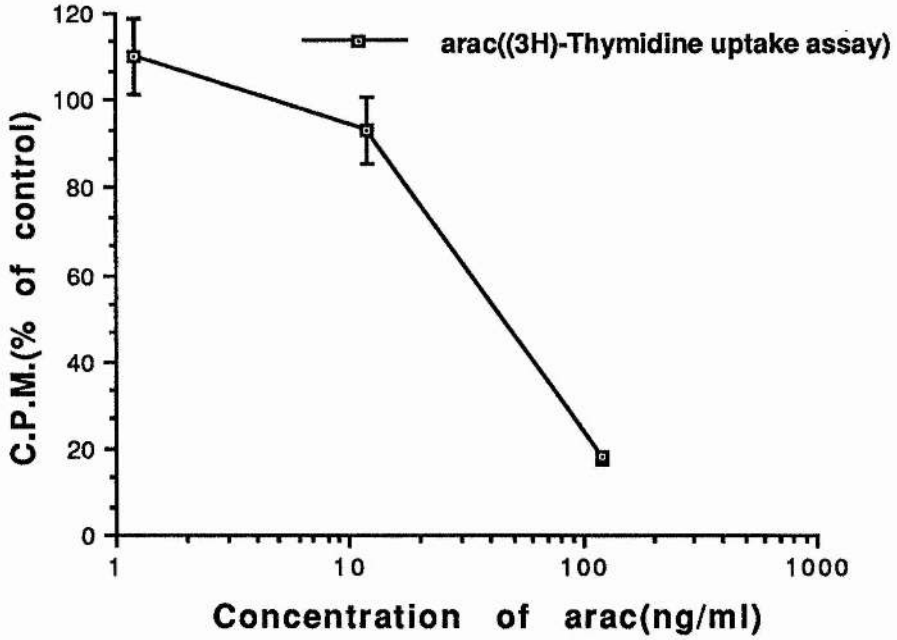


Fig.14: Response of bone marrow cells from 81287T1 cell line to arac. Cytotoxicity was monitored using(3H)- thymidine uptake assay.

Mitoxantrone sensitivity was essentially unchanged up to the sixth passage. An antagonistic effect was seen at low mitoxantrone concentrations (1.2ng/ml) when Ara-C (1.2ng/ml) was added (Figure 17). A comparison of sensitivity of spleen (Figure 18) and bone marrow cells (Figure 16) of the 81287T6 cell line to Ara-C indicates that spleen cells were more sensitive than bone marrow cells to the same concentrations of Ara-C in vitro. Slightly antagonistic to slightly additive effects were observed when mitoxantrone (0.12ng/ml) was added to Ara-C depending on the Ara-C concentration (Figure 18). Similarly, spleen cells (Figure 19) were more sensitive to mitoxantrone at the lowest concentration used as compared to bone marrow cells (Figure 17) of the 81287T6 cell line. By the tenth passage (81287T10), sensitivity of the bone marrow cells to Ara-C was further decreased (Figure 20). Paradoxically however, addition of mitoxantrone (0.27ng/ml) resulted in a synergistic effect. This was so because the extent of [3H]TdR uptake inhibition produced by the combination was greater than the sum of radioactive precursor inhibition produced by the individual drugs. There was little change in response to mitoxantrone even after the tenth passage (Figure 22). Addition of Ara-C (220ng/ml) resulted in an enhanced cytotoxic effect. This was to be expected since Ara-C alone at that concentration (220ng/ml) would inhibit [3H]TdR uptake by 90% relative to control (Figure 21).

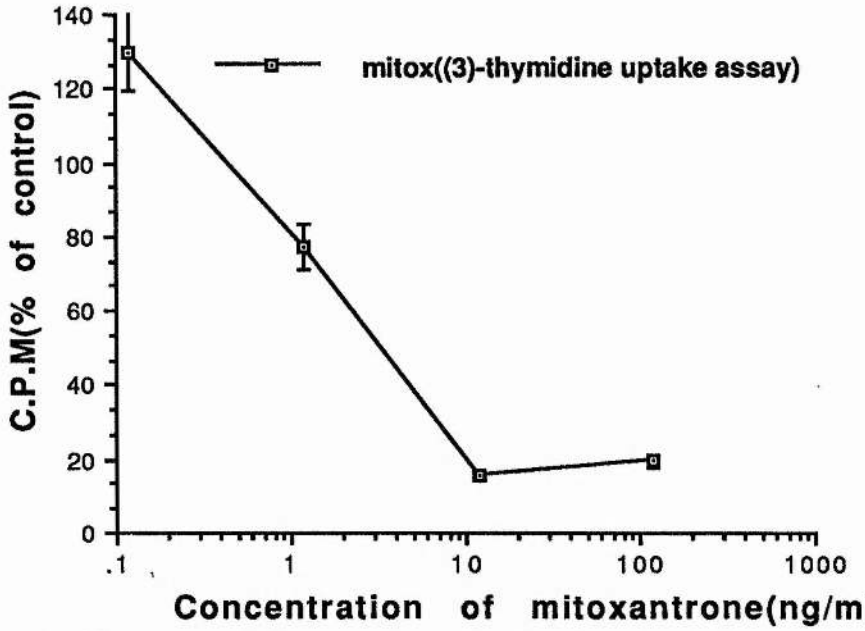


Fig.15: Respose of bone marrow cells from81287T1 cell line to mitoxantrone monitored using(3H)-thymidine uptake assay.

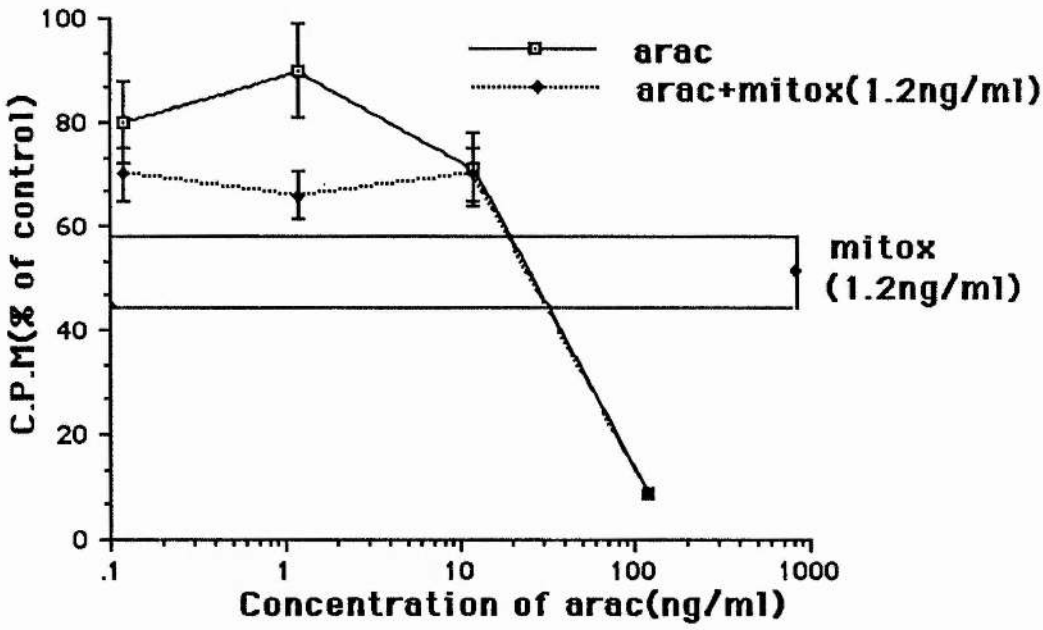


Fig.16: Response of bone marrow cells from 81287T6 cell line to arac alone or in combination with mitoxantrone monitored using the [3H]-thymidine uptake assay.

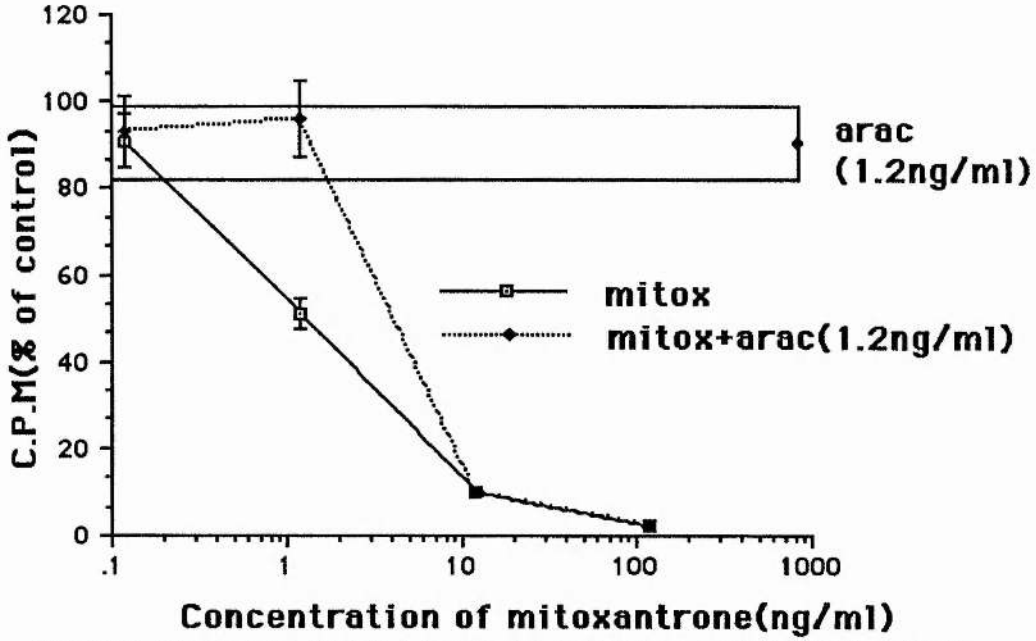


Fig.17: Response of bone marrow cells from 81287T6 cell line to mitoxantrone alone or in combination with arac monitored using (3H)-thymidine uptake assay.

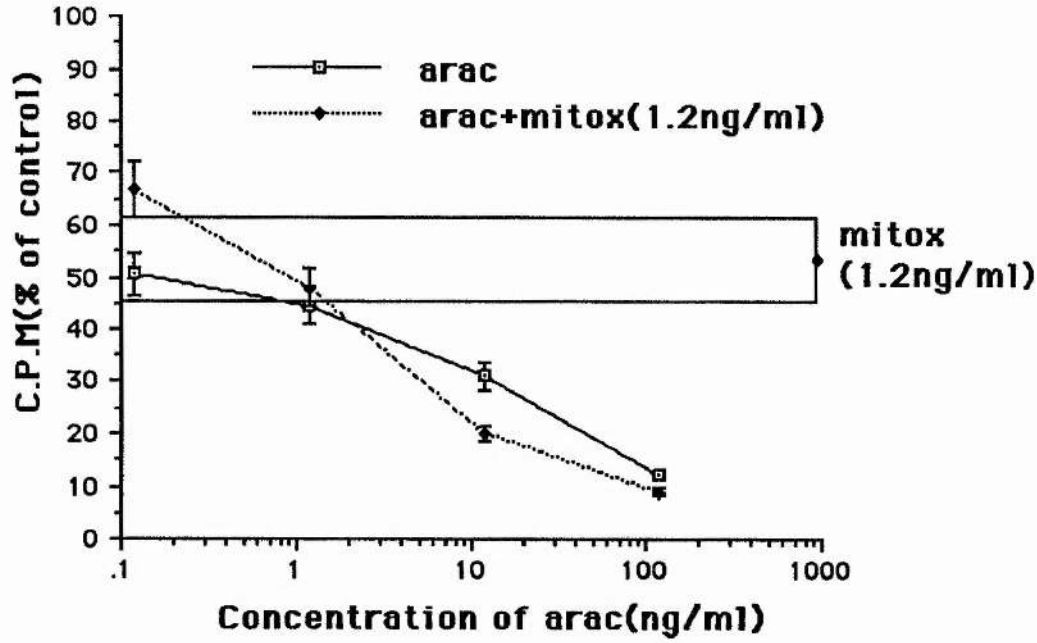


Fig.18: Response of spleen cells from the 81287T6 cell line to arac alone or in combination with mitoxantrone monitored using (3H)-thymidine uptake assay

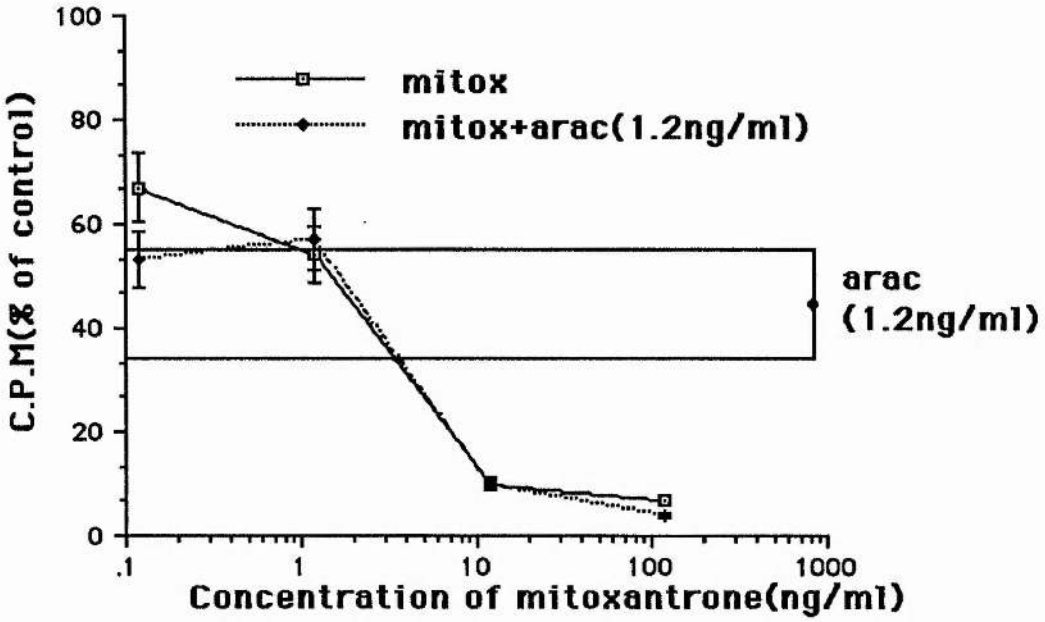


Fig.19.: Response of spleen cells from the 81287T6 cell line to mitoxantrone alone or in combination with arac monitored using (3H)-thymidine uptake assay.

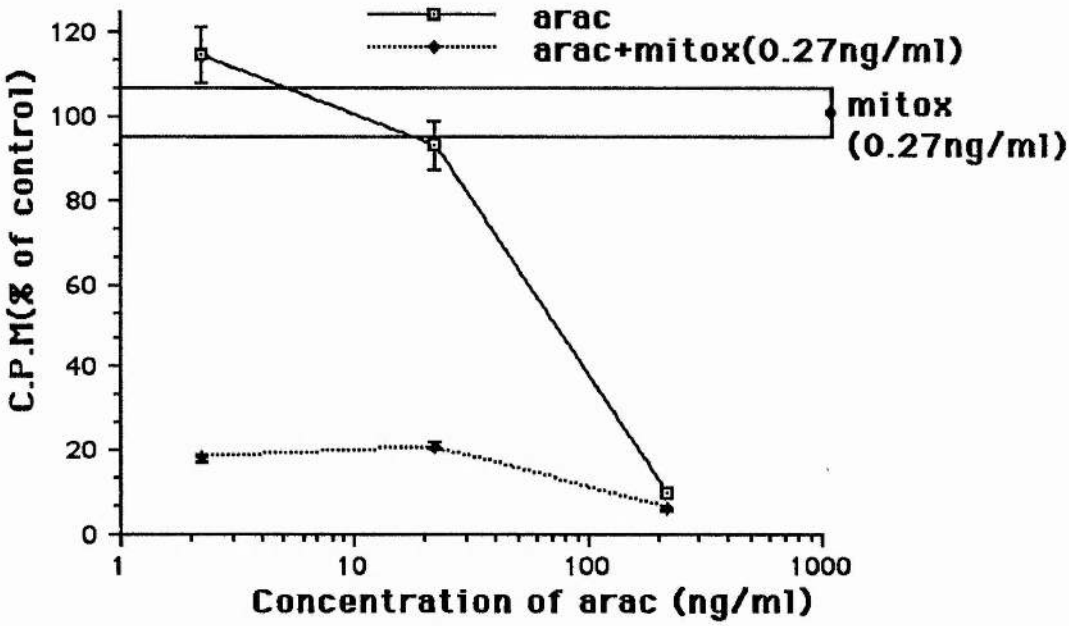


Fig.20: Response of bone marrow cells from 81287T10 cell line to arac alone or in combination with mitoxantrone monitored using (3H)-thymidine uptake assay.

Figure 22 shows the effect of Ara-C alone or in combination with mitoxantrone (0.12ng/ml) on the in vitro proliferation of a pseudo-primary leukaemic cell line 10.12. Although there was a lot of variation in its response to Ara-C, it appears that this cell line was less sensitive than the primary leukaemic cell line 81287 to Ara-C. When mitoxantrone (0.12ng/ml) was added to Ara-C, antagonistic effect was manifested at the lowest Ara-C concentration (1.2ng/ml) while additive effect was observed with intermediate concentration of Ara-C (12ng/ml). The sensitivity of this cell line to mitoxantrone (Figure 23) was similar to that of Ara-C although additive effect was observed using low mitoxantrone concentrations (below 12ng/ml) when Ara-C (0.12ng/ml) was added (Figure 23). Another pseudo-primary cell line (10.13) was not as responsive to Ara-C as 81287 or even the 10.12 cell line. Concentrations of Ara-C below 12ng/ml had little effect on [3H]TdR uptake (Figure 24). When mitoxantrone (0.12ng/ml) was added to Ara-C (0.12-120ng/ml) there was a slightly increased inhibition of [3H]TdR uptake. However, this cell line was more sensitive than 10.12 cell line to mitoxantrone (Figure 25). Mitoxantrone in concentration as low as 0.12ng/ml inhibited [3H]TdR uptake by up to 30% relative to control. When Ara-C (0.12ng/ml) was added to mitoxantrone (0.12-120ng/ml), there was a slight additive effect at the lowest mitoxantrone concentration (0.12ng/ml) and a slight antagonistic effect at 12ng/ml (Figure 25). Thus, the 10.12 cell line was more sensitive to Ara-C than 10.13 cell line while the converse was true for mitoxantrone sensitivity.

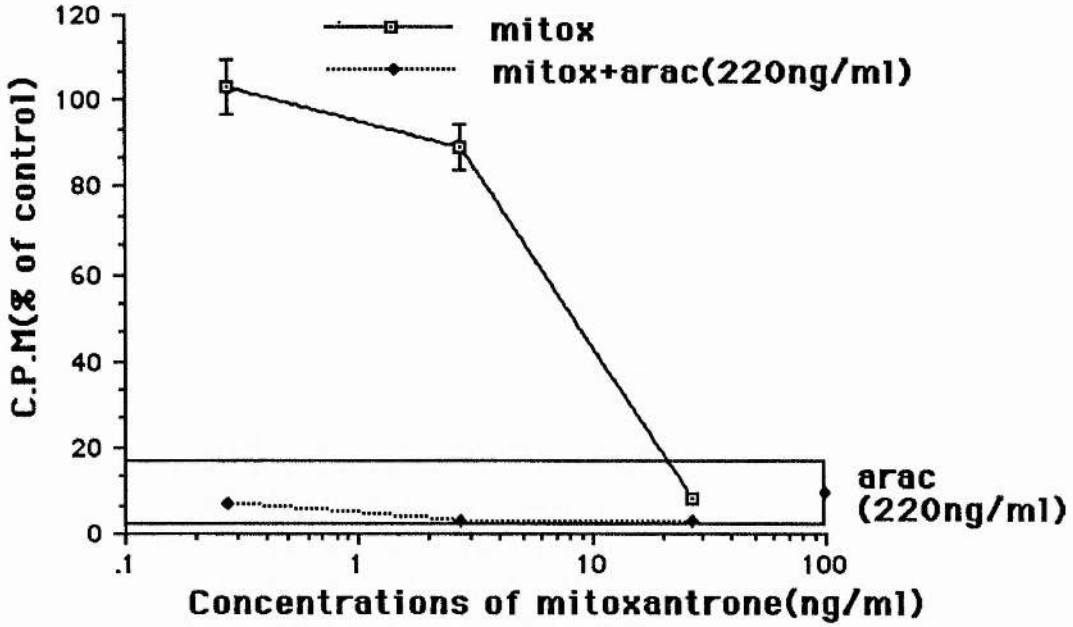


Fig.21: Response of bone marrow cells from 81287T10 cell line to mitoxantrone alone or in combination with arac monitored using (3H)-thymidine uptake assay.

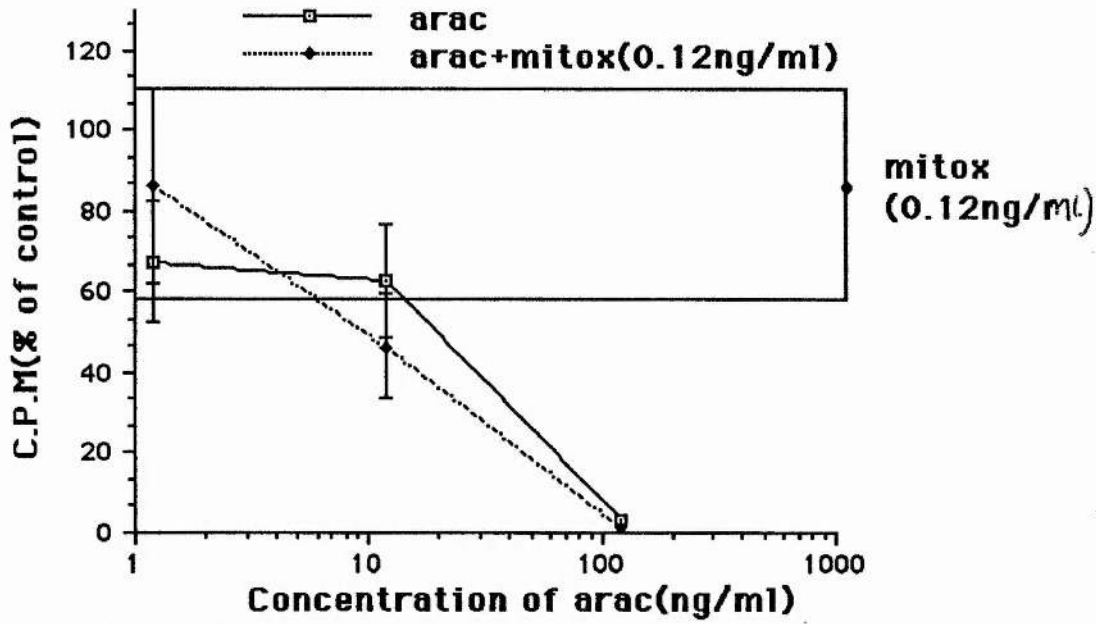


Fig.22: Response of pseudoprimary cell line10.12 to arac alone or in combination with mitoxantrone monitored using(3H)-thymidine uptake assay.

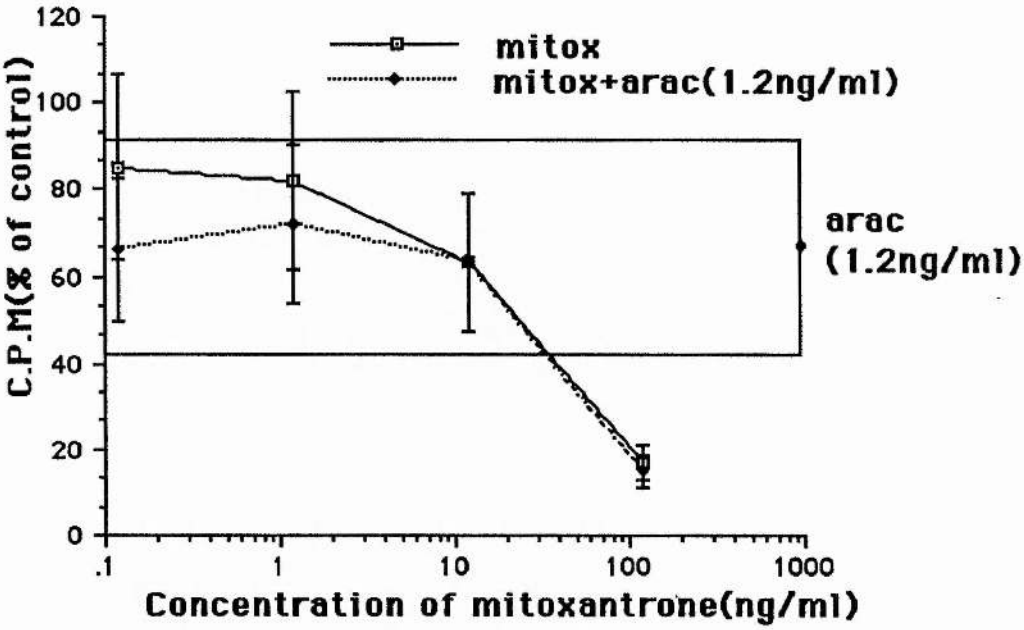


Fig.23: Response of pseudoprimary cell line 10.12 to mitoxantrone alone or in combination with arac monitored using the (3H)-thymidine uptake assay.

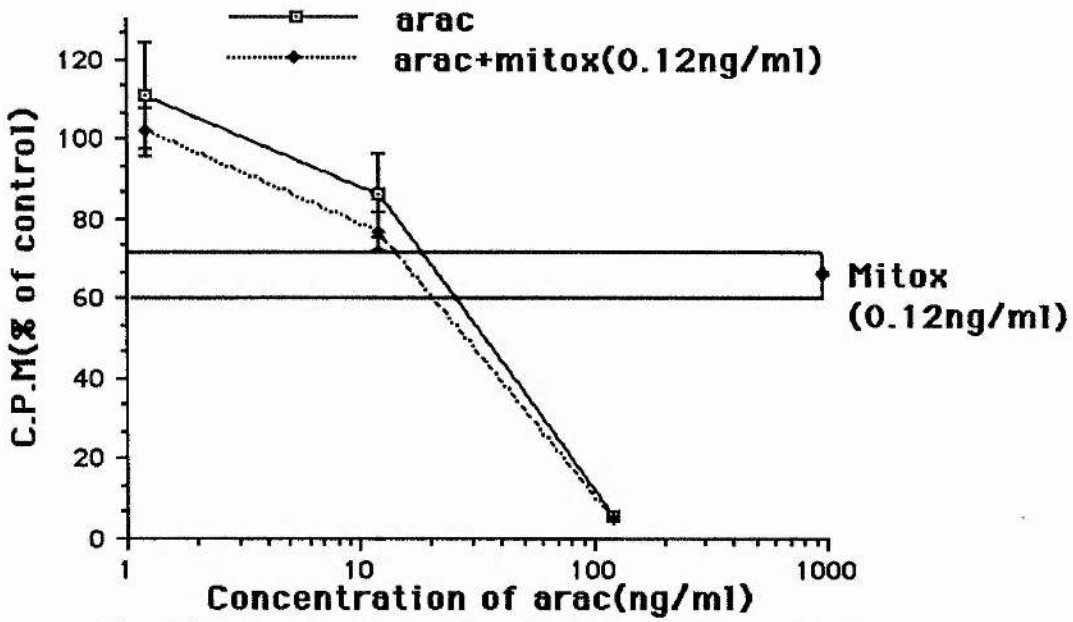


Fig.24:Response of pseudoprimary cell line 10.13 to arac alone or in combination with mitoxantrone monitored using the (3H)-thymidine uptake asssay.

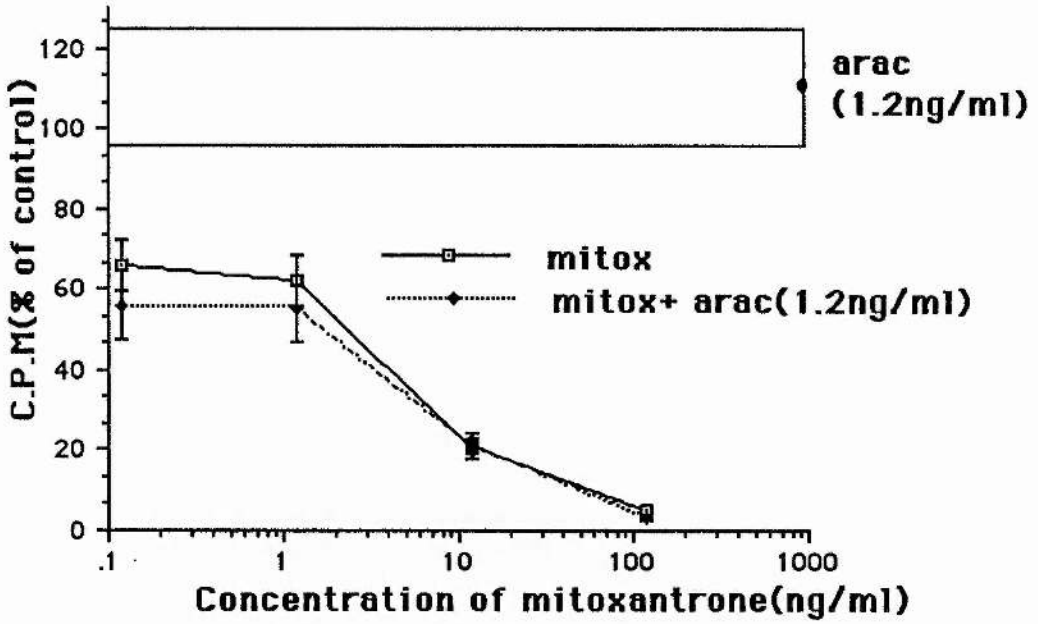


Fig.25: Response of pseudoprimary cell line 10.13 to mitoxantrone alone or in combination with arac monitored using the (3H)-thymidine uptake assay.

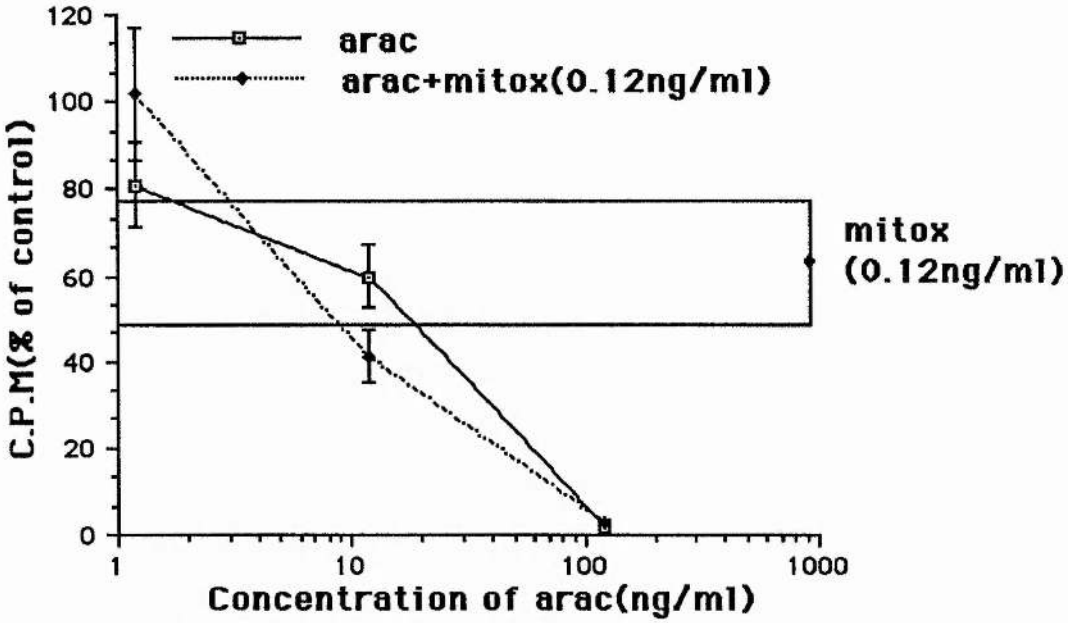


Fig.26: Response of SA7FT5 low cell dose cell line to arac alone or in combination with mitoxantrone monitored using (3H)-thymidine assay.

Low Cell Dose Transplant Cell Lines (SA7FT5; SA8FT14): The responses of the low cell dose transplant cell line SA7FT5 (SA7LD) to Ara-C alone or in combination with mitoxantrone (Figure 26) were similar to those of the pseudoprimary cell line 10.12 (Figure 22). Concentrations of Ara-C below 12ng/ml produced little inhibition of DNA synthesis. When mitoxantrone (0.12ng/ml) was added to Ara-C (1.2-120ng/ml), an antagonistic effect was observed at lowest Ara-C concentration (1.2ng/ml) while slightly additive effect was seen at intermediate concentration (12ng/ml). This low cell dose transplant cell line was more sensitive to mitoxantrone as compared to the pseudoprimary cell line 10.12. A relatively low concentration of mitoxantrone (1.2ng/ml) inhibited DNA synthesis by up to 40% relative to control value (Figure 27). No difference in [3H]TdR uptake inhibition was observed when Ara-C (0.12ng/ml) was added to mitoxantrone. This was to be expected since Ara-C alone at that concentration would produce no effect. The

responses of spleen cells from the SA8FT14 cell line (SA8LD) to Ara-C alone or in combination with mitoxantrone (0.12ng/ml) (Figure 28) and mitoxantrone alone or in combination with Ara-C (Figure 29) were similar to those of the SA7FT5 cell line. Thus, when mitoxantrone (0.12ng/ml) was added to Ara-C, an antagonistic effect was produced using low Ara-C concentration. However, a slightly additive effect was seen when Ara-C concentrations were above 12ng/ml (Figure 28). In contrast when Ara-C (0.12ng/ml) was added to mitoxantrone (0.12ng/ml) an additive effect was manifested at low concentration of mitoxantrone (0.12ng/ml) while no additive cytotoxic effect was seen using intermediate to high concentrations of mitoxantrone (1.2-120ng/ml) (Figure 29).

High Cell Dose Transplant Cell Lines (SA2, SA7 and SA8): Figure 30 shows the dose-response curve of the leukaemic cell line SA2 to Ara-C alone or in combination with mitoxantrone (1.2ng/ml). Dose-response curves reported were the mean of four separate experiments. The SA2 cell line was not sensitive to concentrations of Ara-C below 12ng/ml. An increase in cytotoxicity was manifested when mitoxantrone (1.2ng/ml) was added to Ara-C. However, this was at best an additive effect as mitoxantrone alone at that concentration (1.2ng/ml) would inhibit [3H]TdR uptake to the same extent (Figure 31). The cell line was very sensitive to mitoxantrone (Figure 31) although only slight additive effect was observed when Ara-C (1.2ng/ml) was added to mitoxantrone.

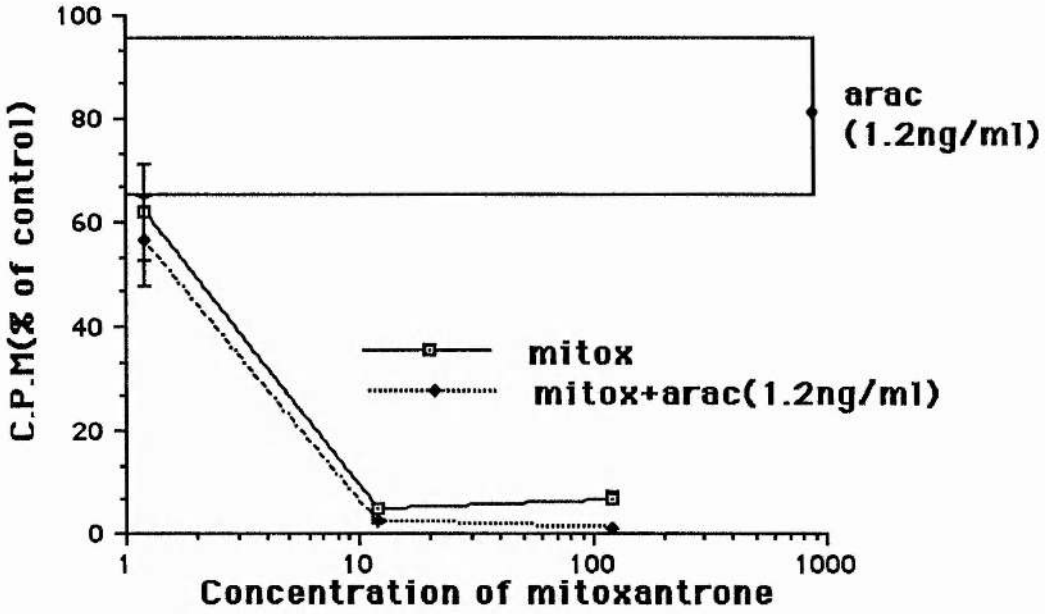


Fig.27: Response of SA7FT5 cell line to mitoxantrone alone or in combination with arac monitored using (3H)-thymidine uptake assay.

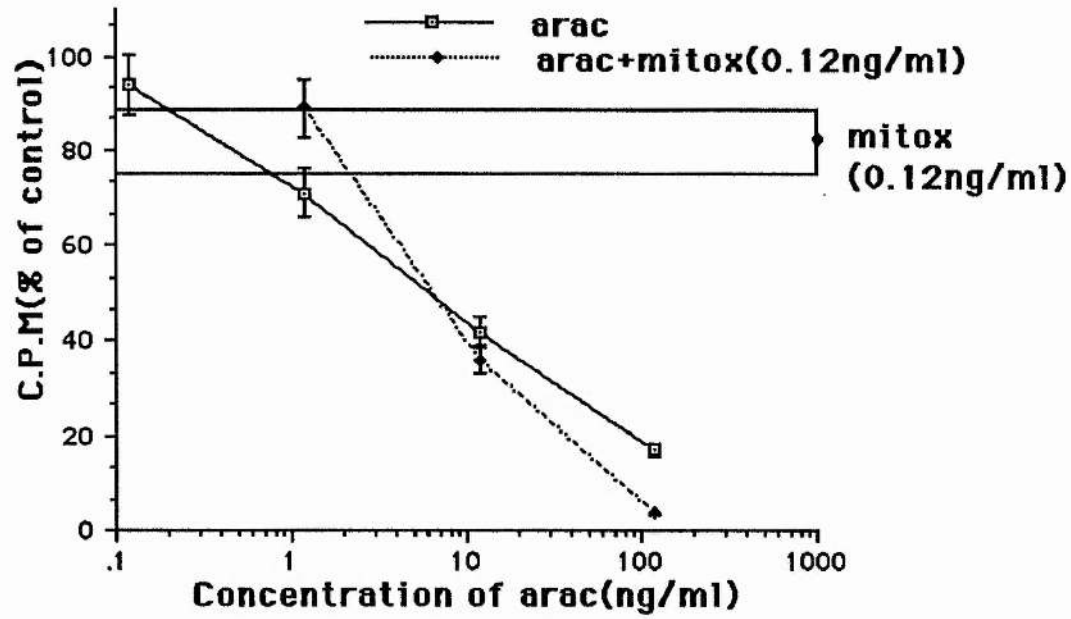


Fig.28: Response of spleen cells from SA8FT14 cell line to arac alone or in combination with mitoxantrone monitored using the (3H)-thymidine uptake assay.

The dose response curves obtained using DiSC (Figure 3) and [3H]TdR uptake assays were not in close agreement except when high concentration (120ng/ml) of mitoxantrone was used. With the DiSC assay and cell number assessment method, shoulders were observed in the dose-response curves of mitoxantrone, whereas in the [3H]TdR uptake assay, the dose-response curve showed simple exponential decline with no apparent shoulder. It may be that the DiSC assay and the cell number assessment method underestimated the extent of cytotoxicity produced by mitoxantrone against SA2 cell line. The SA2 cell line was more sensitive to mitoxantrone alone as compared to Ara-C alone (compare Figures 30 and 31).

The dose-response curves of SA7 high cell dose transplant cell line (SA7HD) following in vitro treatment with Ara-C alone and mitoxantrone alone were plotted from data pooled from 12 separate experiments. The SA7 cell line seem slightly more sensitive to Ara-C alone than SA2 cell line particularly at low Ara-C concentration (0.12-1.2ng/ml). However, it was less sensitive at 12ng/ml. There was a similarity between the dose-response curve of Ara-C determined using the DiSC assay and the [3H]TdR uptake assay. However, whereas an additive effect was observed with the combination of Ara-C and mitoxantrone (1.2ng/ml) in the latter assay, no such effect was seen in the DiSC assay (Figure 4). However, an additive effect was observed for the combination using cell number assessment method. Mitoxantrone seem more potent than Ara-C on the SA7 cell line (Figure 33) and an additive effect was observed when Ara-C (1.2ng/ml) was added to mitoxantrone (Figure 33).

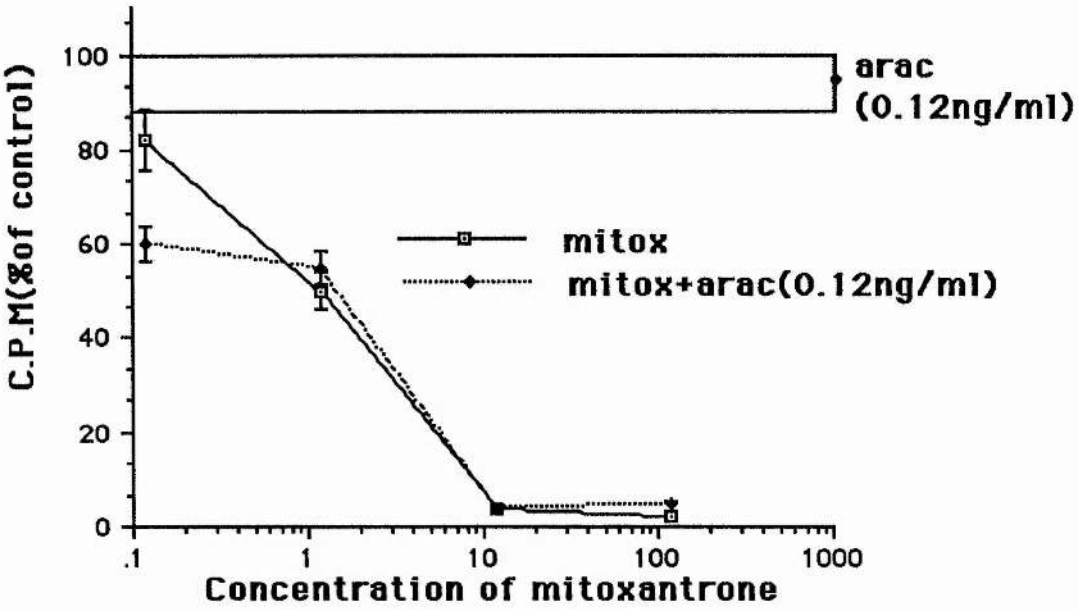


Fig.29: Response of spleen cells from SA8FT14 cell line to mitoxantrone alone or in combination with arac monitored using the (3H)-thymidine uptake assay.

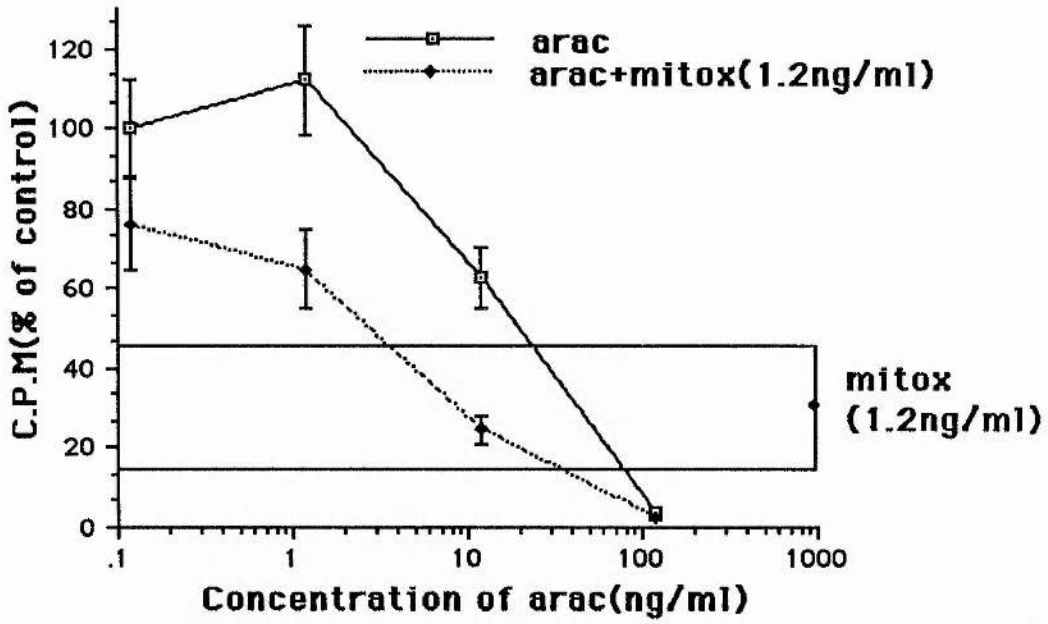


Fig.30: Response of SA2 cell line to arac alone or in combination with mitoxantrone monitored using the (3H)-thymidine uptake assay.

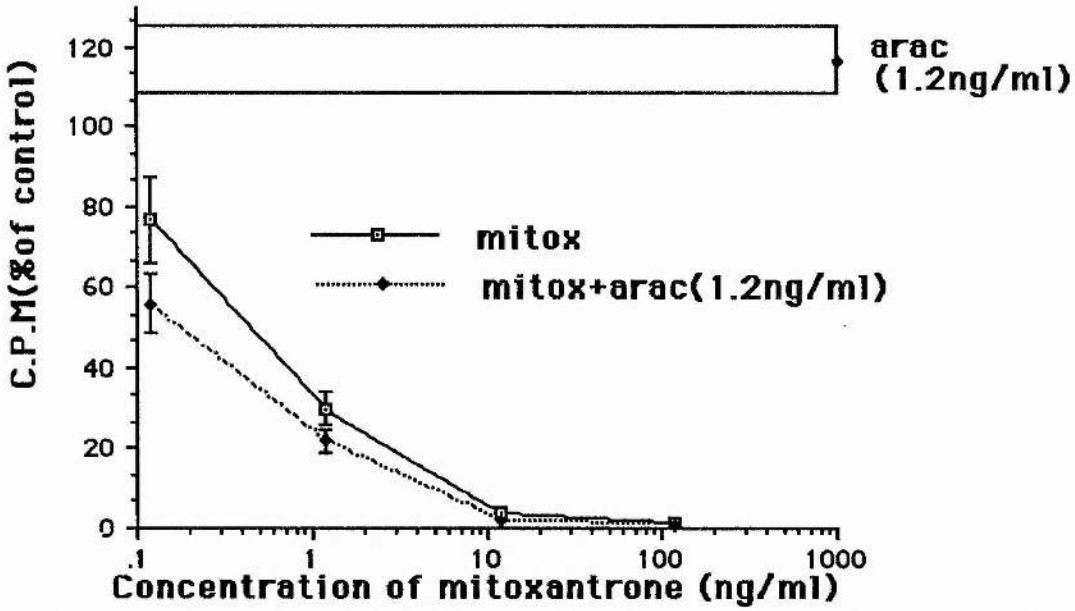


Fig.31:Response of SA2 cell line to mitox alone or in combination with arac monitored using the (3H)-thymidine uptake assay.

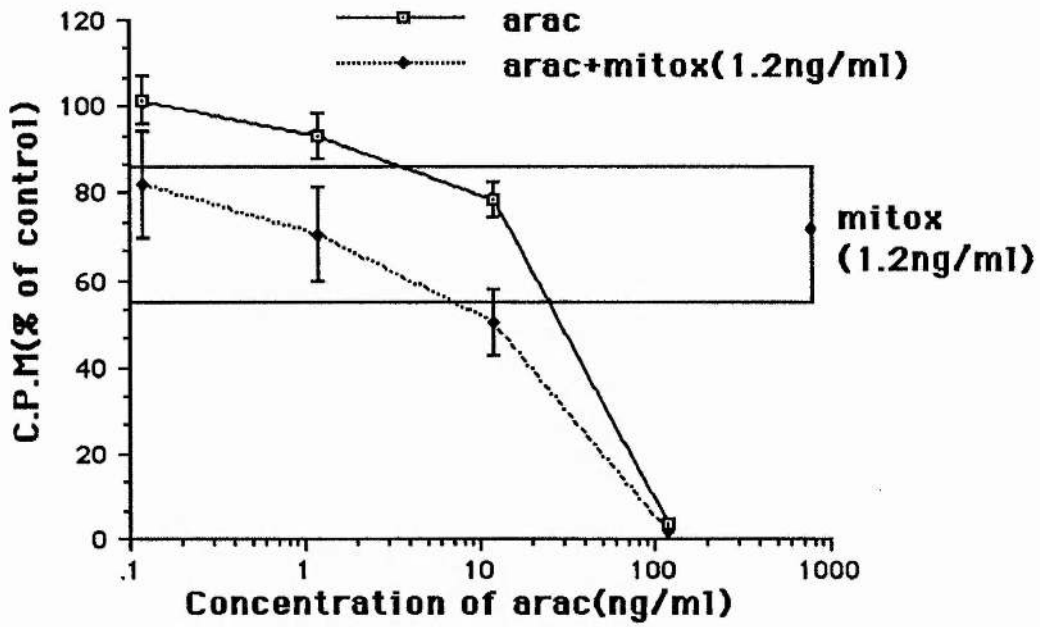


Fig.32: Response of SA7 cell line to arac alone or in combination with mitoxantrone monitored using the (3H)-thymidine uptake assay.

Again, mitoxantrone dose-response curve determined using DiSC assay (Figure 5) was in agreement with the one determined using [3H]TdR uptake assay except with a very low concentration of mitoxantrone (0.12ng/ml). No agreement was seen between the result of the combination experiments using the two methods.

The SA8 high cell dose transplant cell line (Figure 34) was as responsive to Ara-C as SA7HD cell line, although it was more sensitive to the combination of Ara-C with mitoxantrone (1.2ng/ml) as compared to SA7HD. There were similarities in the dose-response curves determined using the cell number assessment method, DiSC assay, (Figure 6) and [3H]-thymidine uptake (Figure 34) assay for both Ara-C alone and in combination with mitoxantrone (Figure 34). The SA8 cell line was more sensitive to mitoxantrone (Figure 35) as compared to SA7 cell line (Figure 33) and when Ara-C (1.2ng/ml) was added, there was only a slight increase in the extent of [3H]TdR uptake inhibition.

Normal murine bone marrow cells (NBM) seem slightly more sensitive than murine leukaemic cell lines SA2, SA7HD and SA8HD to Ara-C particularly in the concentration range 0.12-1.2ng/ml (Figure 36). Dose-response curves of normal bone marrow cells were constructed using pooled data from at least 5 separate experiments. NBM cells seem more sensitive than leukaemic cell lines SA2, SA7HD and SA8HD to the combination of Ara-C with mitoxantrone (1.2ng/ml) (Figure 36). In addition, they were as sensitive to mitoxantrone as SA7HD and SA8HD.

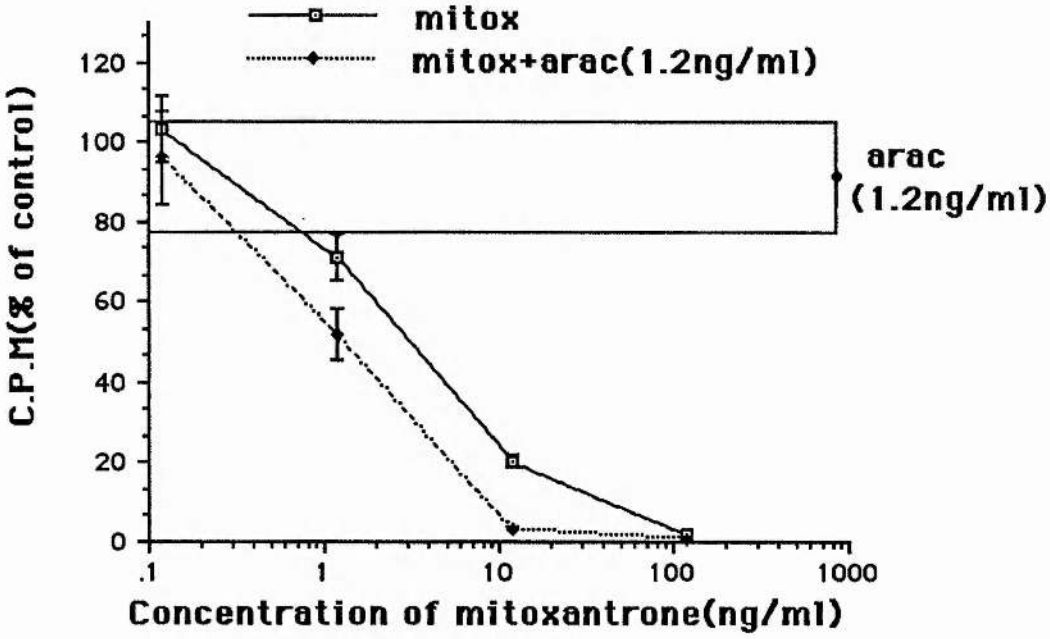


Fig.33: Response of SA7 cell line to mitoxantrone alone or in combination with arac monitored using the (3H)-thymidine uptake assay.

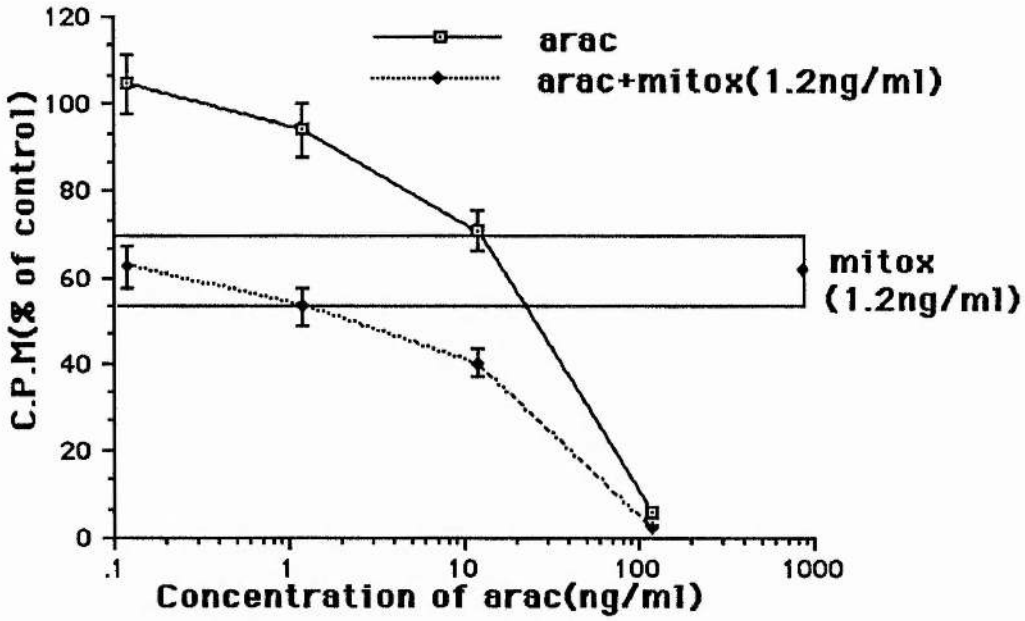


Fig.34: Response of SA8 cell line to arac alone or in combination with mitoxantrone monitored using the (3H)-thymidine uptake assay.

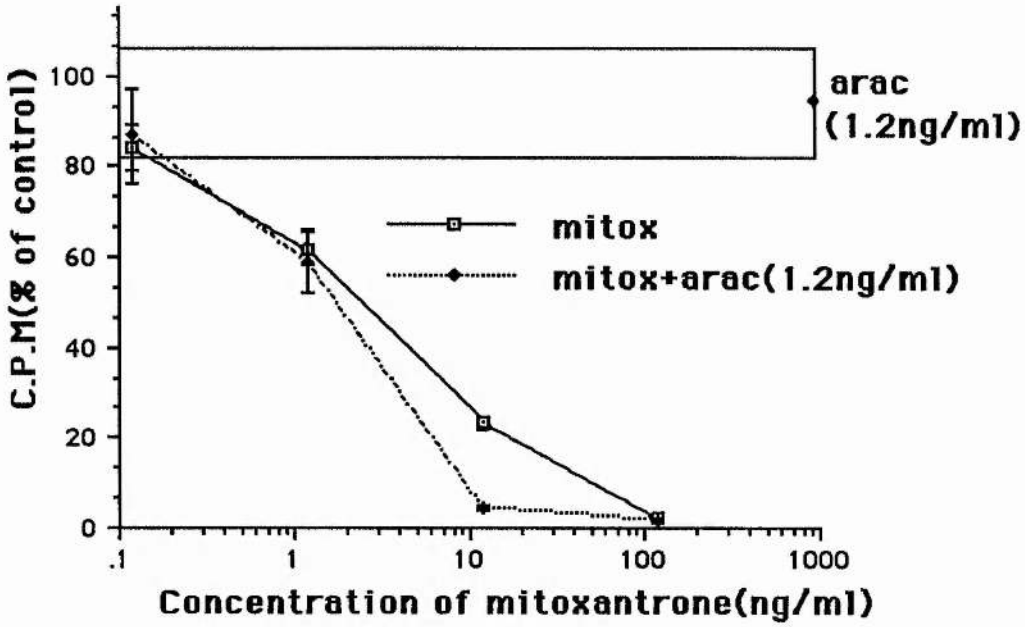


Fig.35: Response of SA8 cell line to mitoxantrone alone or in combination with arac monitored using the (3H)-thymidine uptake assay.

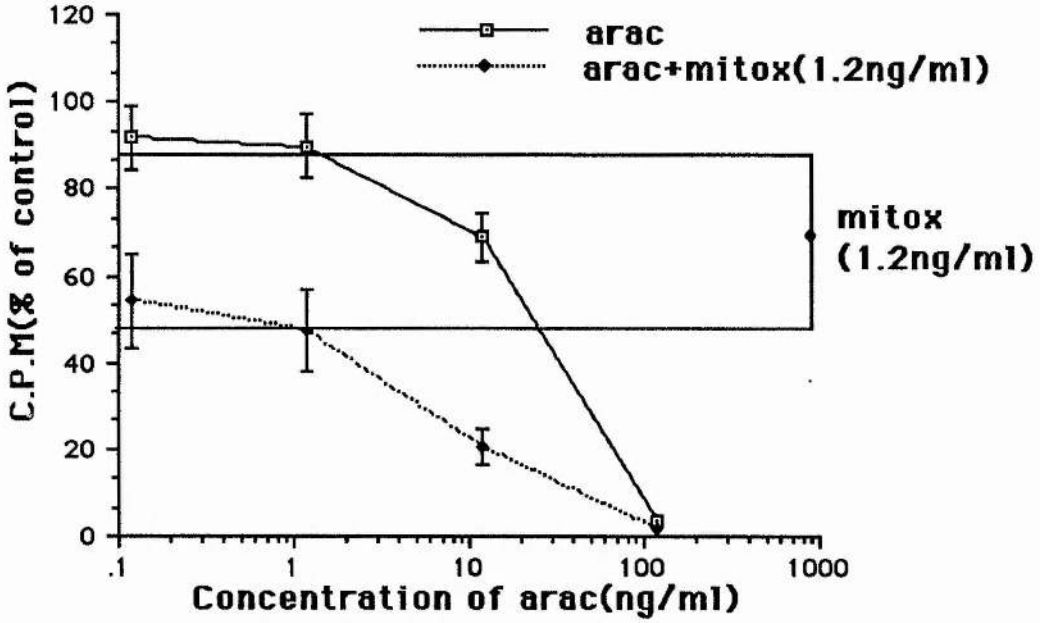


Fig.36: Response of normal bone marrow cells (NBM) to arac alone or in combination with mitoxantrone monitored using the (3H)-thymidine uptake assay.

However, they were less sensitive than these cell lines to combination of mitoxantrone and Ara-C (1.2ng/ml) at low mitoxantrone concentration (0.12-1.2ng/ml)(Fig.37).

Of the leukaemic cell lines studied, the SA2 was most responsive to continuous incubation with mitoxantrone, followed by SA8 and SA7 in that order. Similarly the SA2 was more sensitive than SA7 (but not SA8) to combination of Ara-C with mitoxantrone. As compared to NBM cells, both SA2 and SA7 were more sensitive to combination of mitoxantrone and Ara-C (1.2ng/ml).

3.2.4 Scheduling Studies: In these in vitro experiments, either normal bone marrow cells or leukaemic bone marrow cells from SA2, SA7HD and SA8HD were pulsed with a drug for one hour, washed twice and a second drug was added either immediately after washing or 24, 48 or 72 hours later. The results of one hour pulse with Ara-C followed by addition of mitoxantrone (section 3.2.4.1) are reported first, followed by those of one hour pulse with mitoxantrone followed by addition of Ara-C (section 3.2.5.2).

3.2.4.1 One Hour Pulse with Ara-C Followed by Addition of Mitoxantrone:

Normal bone marrow cells or leukaemic bone marrow from the cell lines SA2, SA7HD and SA8HD were either pulsed with Ara-C in the concentration range 0.12-120ng/ml or Ara-C (same concentration range) together with mitoxantrone (1.2ng/ml) for one hour. The cells were then washed twice, counted and plated at 7×10^4 cells per well in a microtitre plate. Mitoxantrone (1.2ng/ml) was added (to those wells containing cells that were pulsed with Ara-C only) either immediately following cell wash or 24, 48 or 72 hours later (Figure 38). WEHi

conditioned medium was added (to give a final concentration of 10% per well) and the plates were incubated for four days. Control cells were pulsed with medium only. Plates were then labelled with [3H]TdR, harvested and the amount of radioactivity incorporated by the cells was determined (Figure 38).

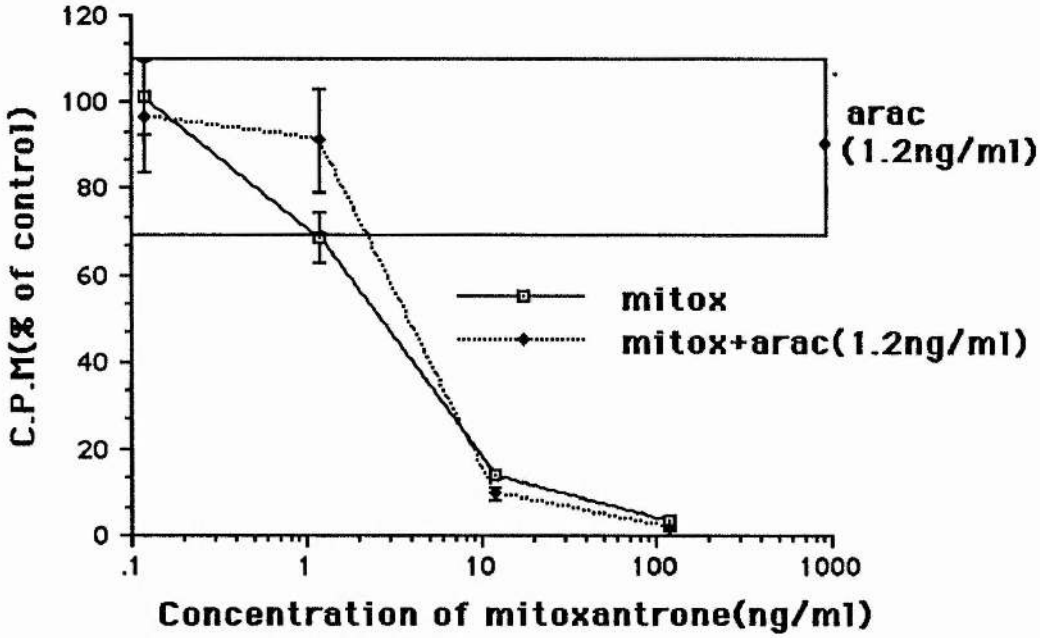
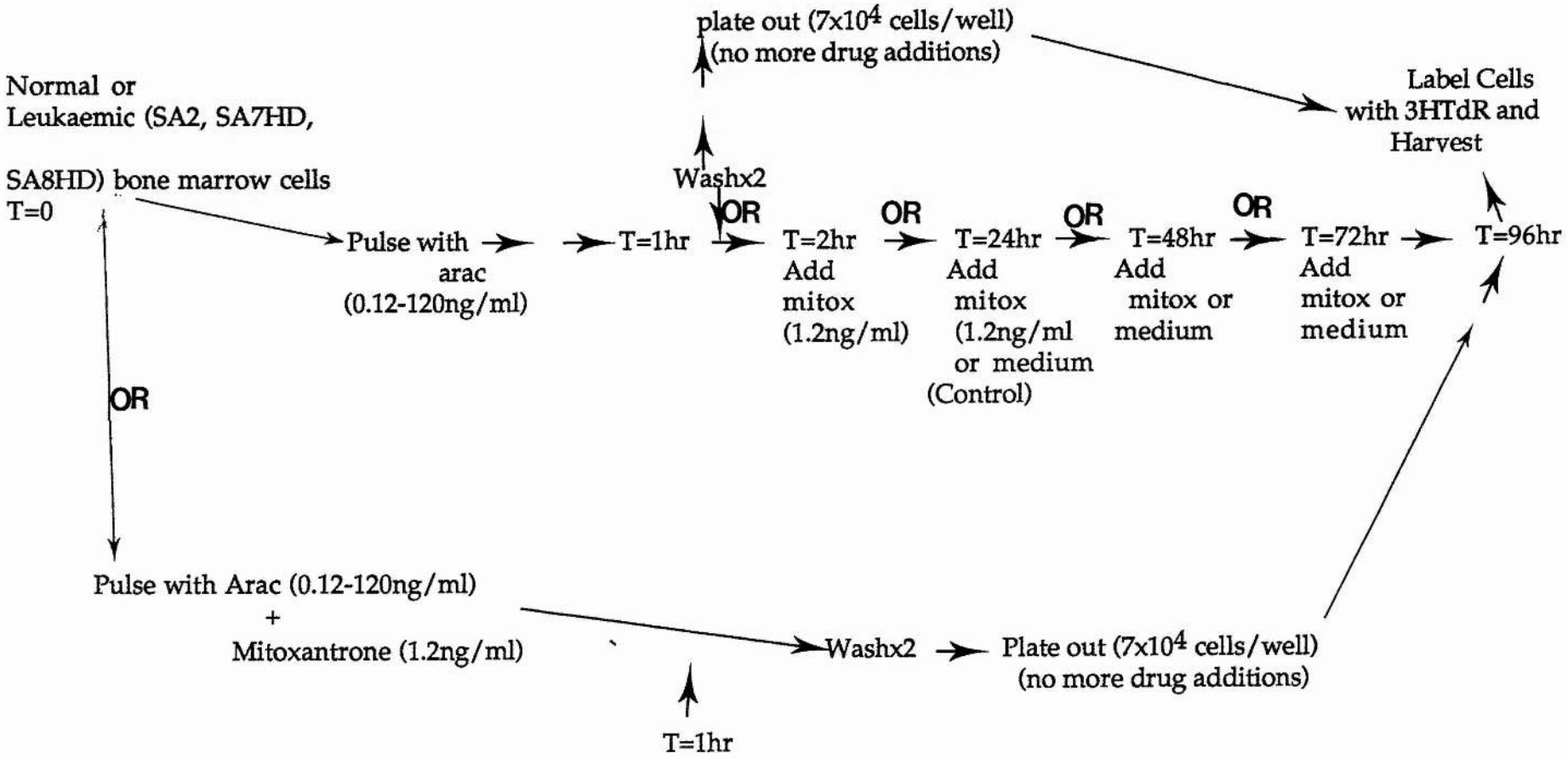


Fig.37: Response of normal bone marrow cells to mitoxantrone or in combination with arac monitored using the [3H] thymidine uptake assay.

Figure 38 : Flow diagram of one-hour pulse with arac alone or in combination with mitoxantrone followed by the addition of mitoxantrone (mitox) either immediately after washing the cells or 24, 48 or 72 hrs later.



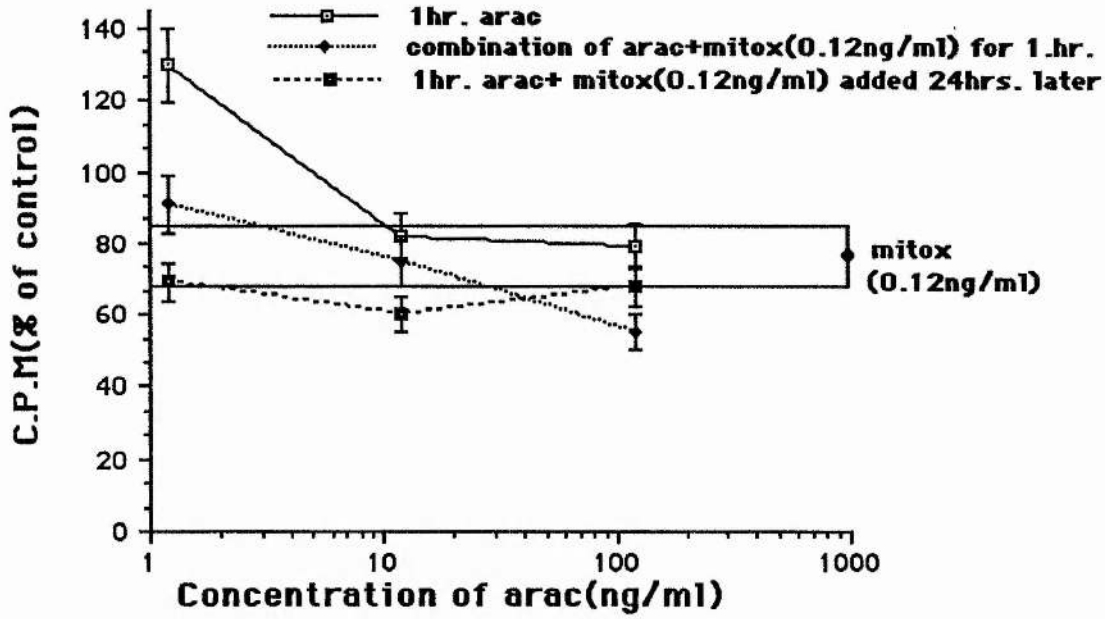


Fig.39: Response of SA2 cell line to 1hr. pulse with arac alone; 1hr.pulse with both arac and mitox; or 1hr. pulse with arac with mitoxantrone added 24hrs.later.

Results: Figure 39 shows the responses of SA2 cell line to one hour pulse with Ara-C alone or in combination with mitoxantrone (0.12ng/ml) as compared to one hour pulse with Ara-C with mitoxantrone added to the cells 24 hours later. A one hour pulse with both Ara-C (0.12-120ng/ml) and mitoxantrone (0.12ng/ml) produced an additive effect as compared to a one hour pulse with Ara-C alone. However, there was no difference between one hour pulse with both drugs and one hour pulse with Ara-C followed by addition of mitoxantrone 24 hours later (Figure 39). The latter schedule was still more effective at inhibiting [3H]TdR uptake as compared to one hour pulse with Ara-C alone. There was no difference between one hour pulse with Ara-C followed immediately by addition of mitoxantrone and one hour pulse with Ara-C with mitoxantrone added 48 hours later (Figure 40). Both schedules were not as effective as pulsing the cells with

the two drugs for one hour. As expected, there was more pronounced inhibition of [3H]TdR uptake when mitoxantrone was added 24 hours rather than 48 or 72 hours following one hour pulse with Ara-C (Figure 41). Thus, the longer mitoxantrone remained in culture, the more pronounced was its effect on [3H]TdR uptake.

Unlike what was observed with SA2 cell line, scheduling had a marked effect on inhibition of [3H]TdR uptake produced by Ara-C and mitoxantrone on the SA7 high cell dose transplant cell line. Thus, there was a synergistic effect on DNA synthesis inhibition when mitoxantrone was added 24 hours rather than immediately following one hour pulse with Ara-C (Figure 42). Virtually no additive effect was observed when both Ara-C (0.12-120ng/ml) and mitoxantrone (1.2ng/ml) were pulsed for one hour. Adding mitoxantrone as late as 72 hours following one hour pulse with Ara-C exerted a greater effect on [3H]TdR uptake as compared to when cells were pulsed with both drugs for one hour (Figure 43). Maximal inhibition was seen when mitoxantrone was added 24 hours following one hour pulse with Ara-C and decreasing effect if mitoxantrone was added 48 or 72 hours later (Figure 43). The least effective schedule was when cells were pulsed with both drugs for one hour.

Scheduling had no effect on one hour pulse with Ara-C followed by mitoxantrone using the SA8 cell line. There was no difference between one hour pulse with Ara-C alone and one hour pulse with Ara-C followed by addition of mitoxantrone (1.2ng/ml) 24 hours later (Figure 44). One hour pulse with Ara-C followed by the addition of mitoxantrone immediately was only slightly more effective as compared to when mitoxantrone was added 24 hours later. One hour pulse with both drugs was not more effective than 1 hour pulse with Ara-C alone

(Figure 45), and when mitoxantrone was added 24, 48 or 72 hours later, there was increased [3H]TdR uptake (Figure 46).

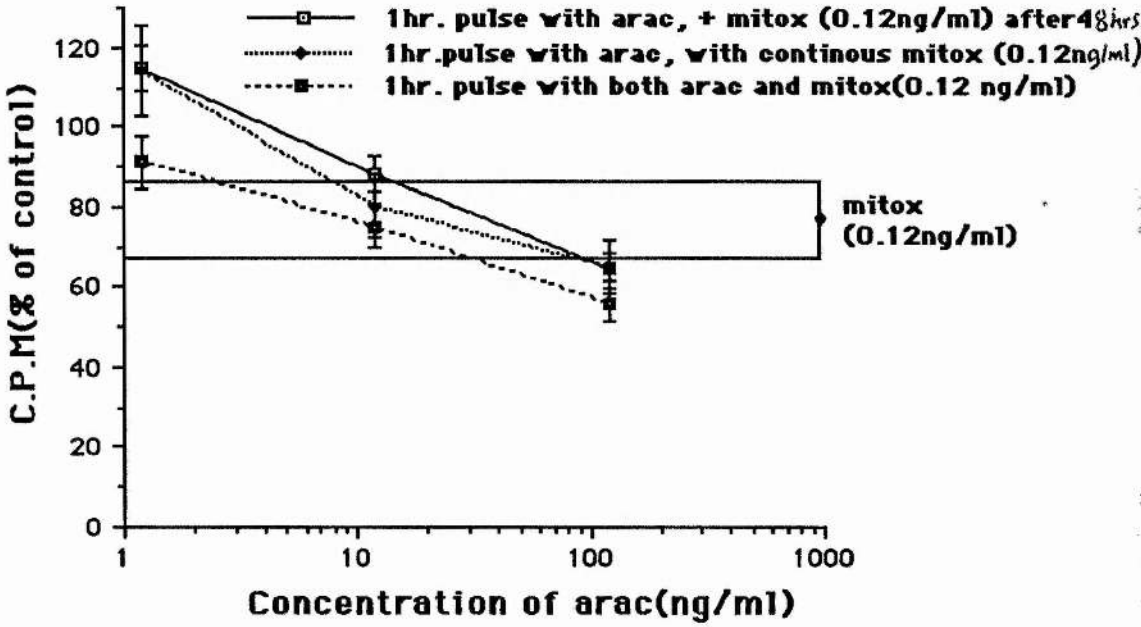


Fig.40: Response of SA2 cell line to 1hr. pulse with arac alone; 1hr. pulse with arac followed by immediate additon of mitoxantrone; or 1hr. pulse with both arac and mitoxantrone.

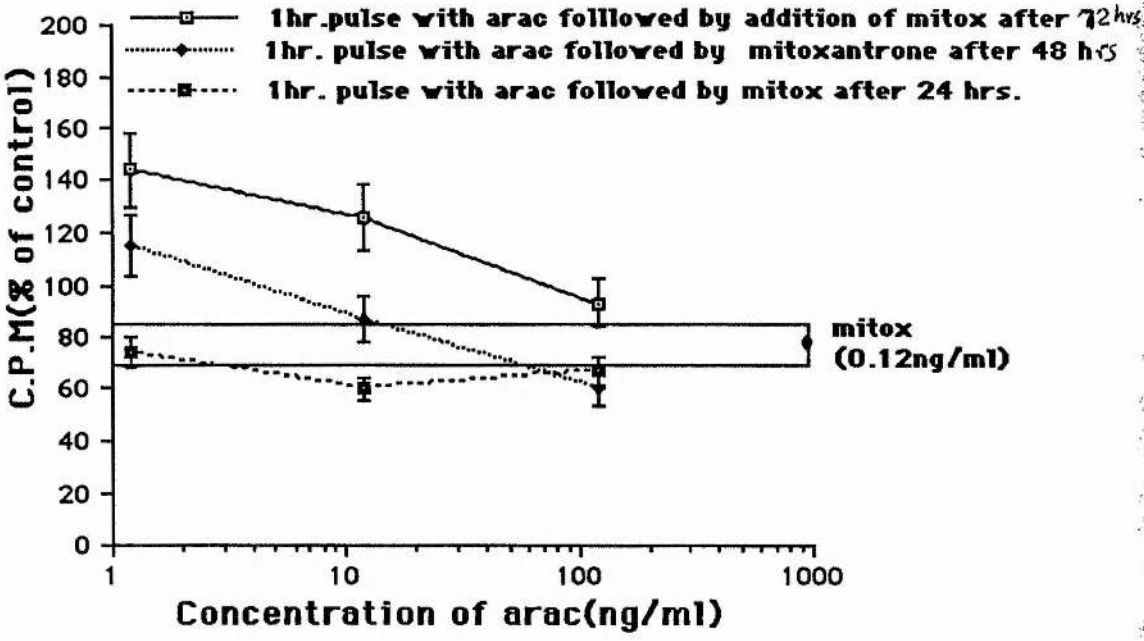


Fig.41 : The effect of 1hr. pulse with arac followed by the addition of mitoxan (0.12ng/ml) either 24, 48, or 72hrs. later on the SA2 cell line.

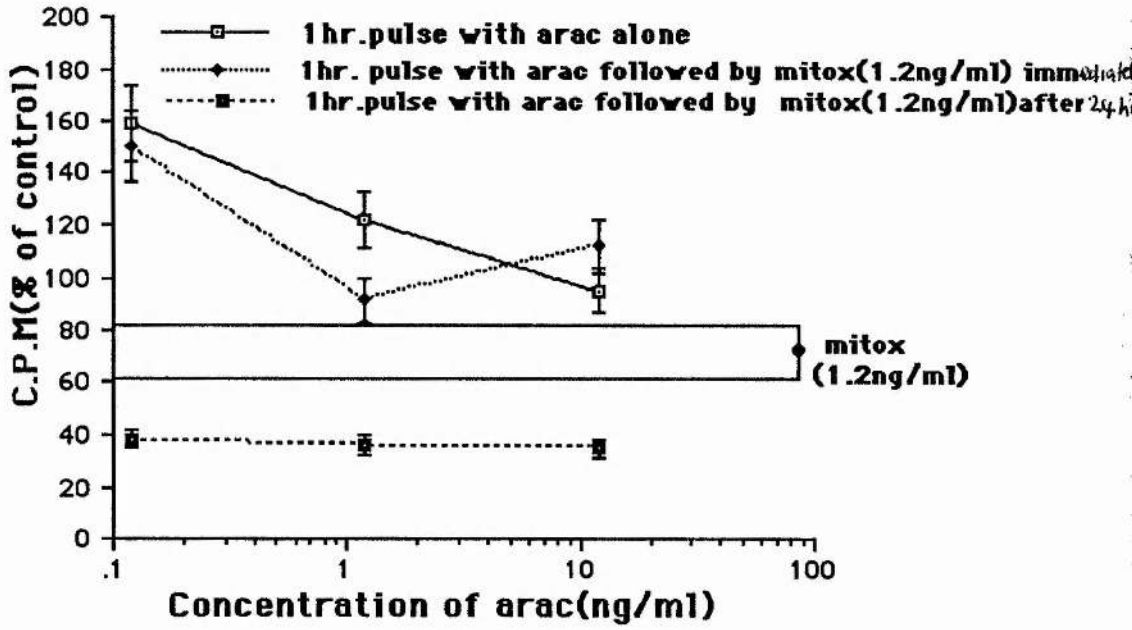


Fig.42: The effect of 1hr. pulse with arac alone;1hr. pulse with arac, followed immeditely by the addition of mitox;1hr. pulse with arac, followed by the addition of mitox after 24hrs. on the SA7 cell line.

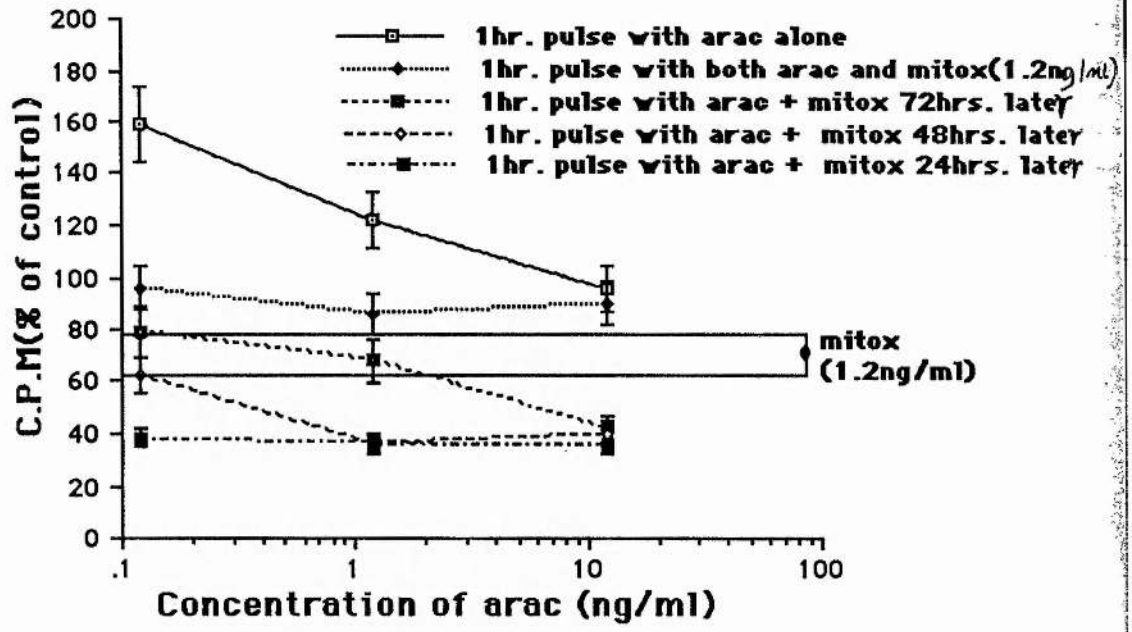


Fig.43:The effect of 1hr. pulse with arac alone ; 1hr. pulse with arac followed by the addition of mitox(1.2ng/ml) either 24,48,or72 hrs. later on SA7 cell line.

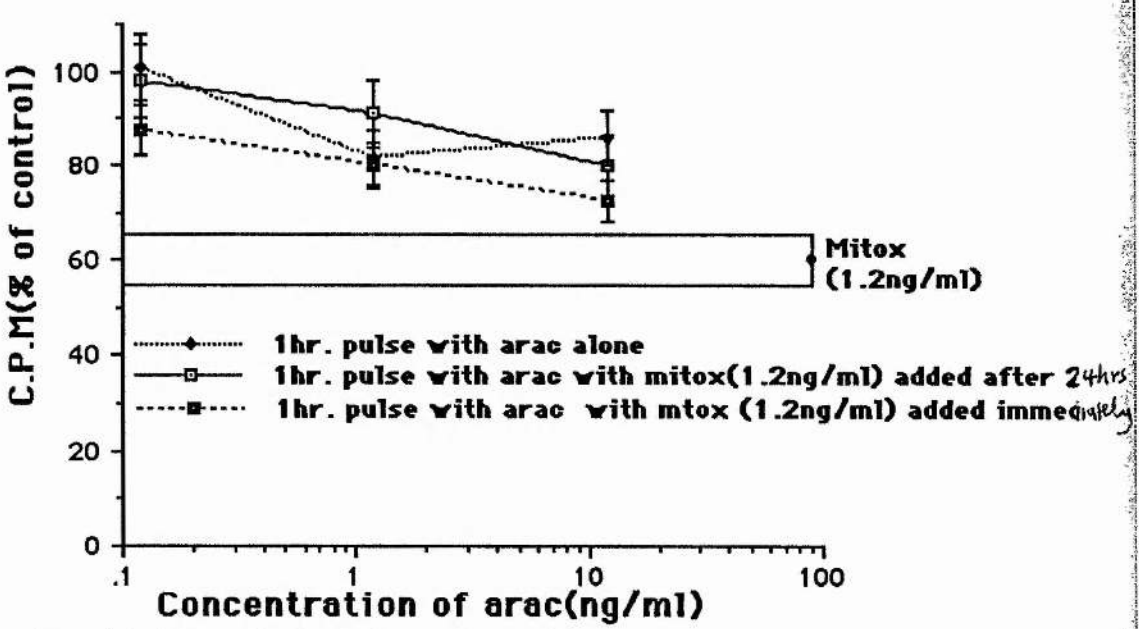


Fig.44: The effect of 1hr. pulse with arac alone ; 1hr. pulse with arac, followed by the addition of mitox either immediately, or 24 hrs. later , on the SA8 cell line.

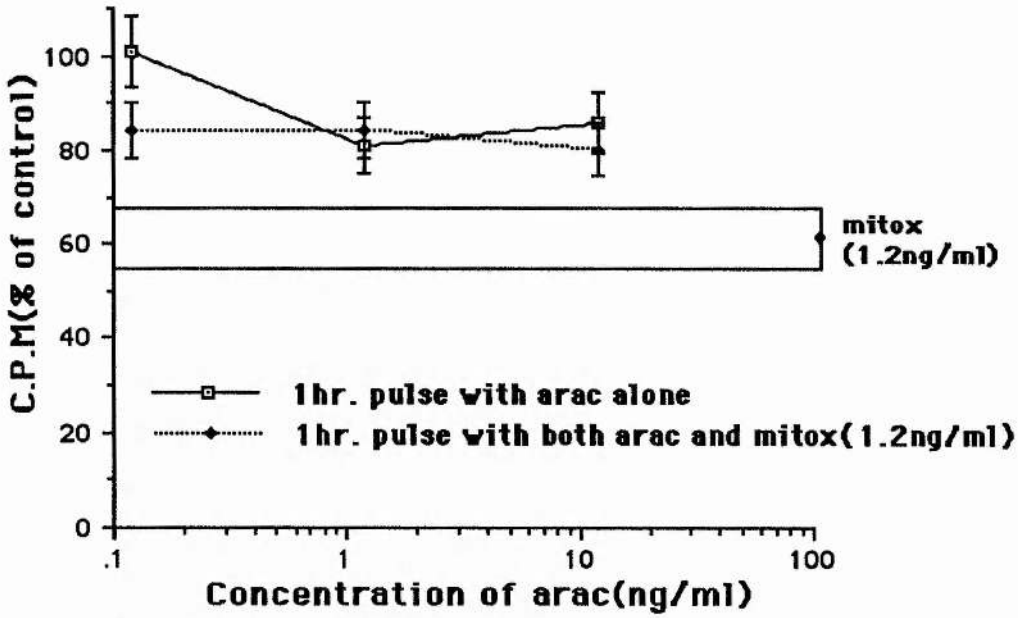


Fig.45: The response of SA8 cell line to 1hr. pulse with arac alone or in combination with mitoxantrone monitored using the (3H)-thymidine uptake assay.

As was observed with SA7 cell line, scheduling had a marked effect on the cytotoxicity produced by one hour pulse with Ara-C followed by addition of mitoxantrone in normal bone marrow cells. One hour pulse with Ara-C (0.12-120ng/ml) followed by addition of mitoxantrone 24 hours later was more effective at inhibiting [3H]TdR uptake as compared to when mitoxantrone was added immediately after pulsing with Ara-C (Figure 47). One hour pulse with both drugs was no more effective than one hour pulse with Ara-C alone. When mitoxantrone (0.12ng/ml) was added immediately after one hour pulse with Ara-C (Figure 47), there was an increase in [3H]TdR uptake which was more pronounced at an Ara-C concentration of 1.2ng/ml. As expected, mitoxantrone was more effective at inhibiting [3H]TdR uptake if it was added 24 hours rather than 48 or 72 hours following one hour pulse with Ara-C (Figure 48). An increased inhibition of [3H]TdR uptake was seen when Ara-C was left

in culture (continuous incubation) and mitoxantrone (1.2ng/ml) was added 24, 48 or 72 hours later (Figure 49).

3.2.4.2 One hour Pulse with Mitoxantrone Followed by Addition of AraC: In these experiments normal bone marrow cells or leukaemic bone marrow cells (SA2, SA7 and SA8) were pulsed with mitoxantrone (0.12-120ng/ml) for one hour, followed either immediately after washing the cells or 24, 48 or 72 hours later by the addition of Ara-C (1.2ng/ml) (Figure 50).

Results: A one hour pulse with mitoxantrone (Figure 51) resulted in greater inhibition of DNA synthesis as compared to one hour pulse with Ara-C (Figure 39) on the SA2 cell line. An additive affect which was more pronounced at low mitoxantrone concentration (0.12ng/ml) was seen when SA2 leukaemic cells were pulsed with both drugs for one hour (Figure 51). A one hour pulse with both drugs was more effective at inhibiting [3H]TdR uptake as compared to when Ara-C was added either immediately or 24 hours following one hour pulse with mitoxantrone (Figure 52). Greater [3H]TdR uptake inhibition was seen when Ara-C was added 24 hours rather than immediately following one hour pulse with mitoxantrone (Figure 52). Whether Ara-C was added 24, 48 or 72 hours following one hour pulse with mitoxantrone made no difference except at low mitoxantrone concentration (0.12ng/ml) when the extent of [3H]TdR uptake inhibition depended on the time Ara-C was added (Figure 53).

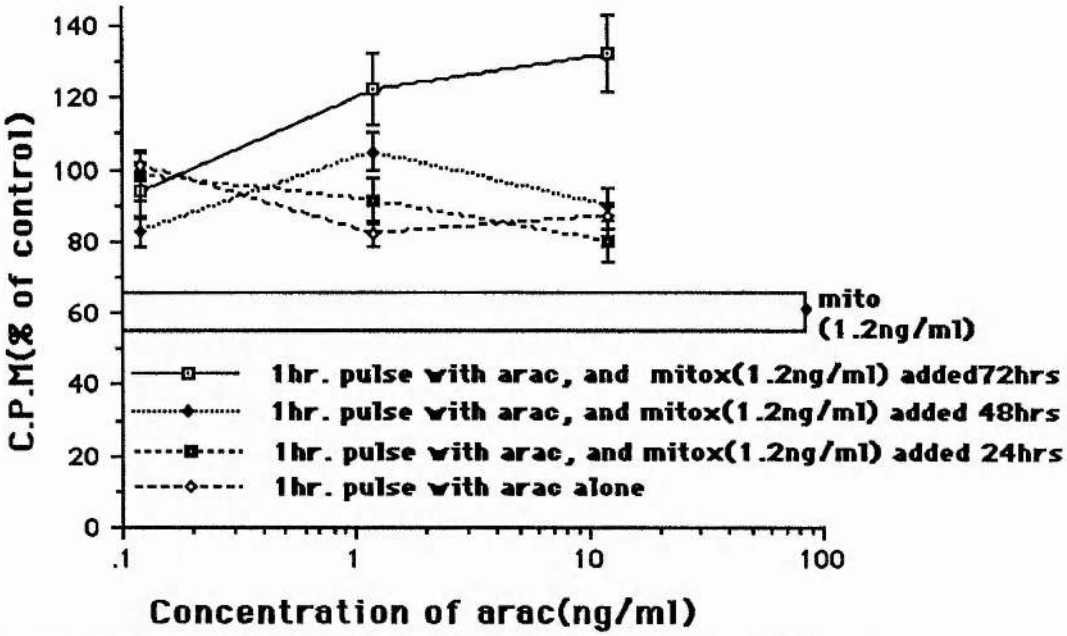


Fig.46: The response of SA8 cell line to 1hr. pulse with arac alone or with mitox added 24, 48 or 72 hrs later. Cytotoxicity was monitored using the (3H)-thymidine uptake assay.

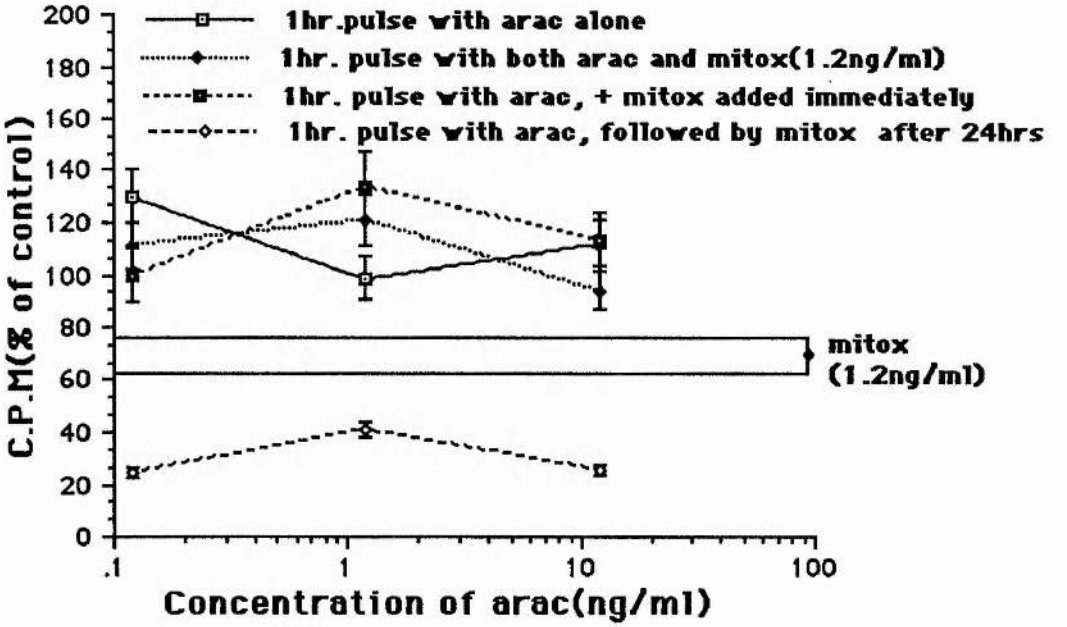


Fig.47: The response of NBM cells to 1hr. pulse with arac; 1hr. pulse with both arac and mitox; or 1hr. pulse with arac, followed by mitox(1.2ng/ml) either immediately or 24 hrs later.

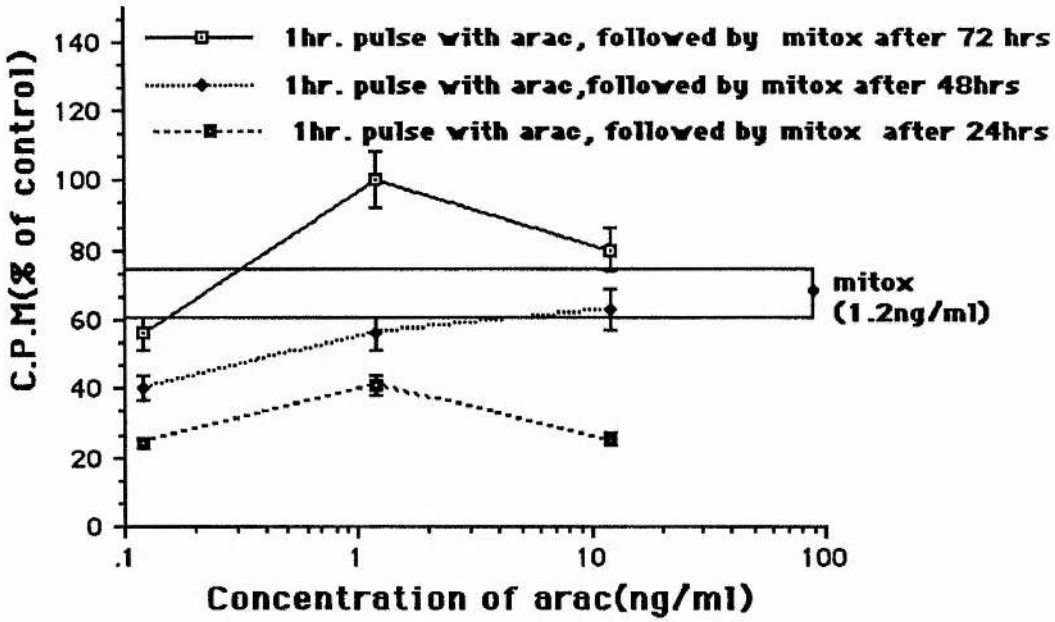


Fig.48: The response of NBM cells to 1hr. pulse with arac followed by the addition of mitox(1.2ng/ml) after 24, 48, or 72hrs later.

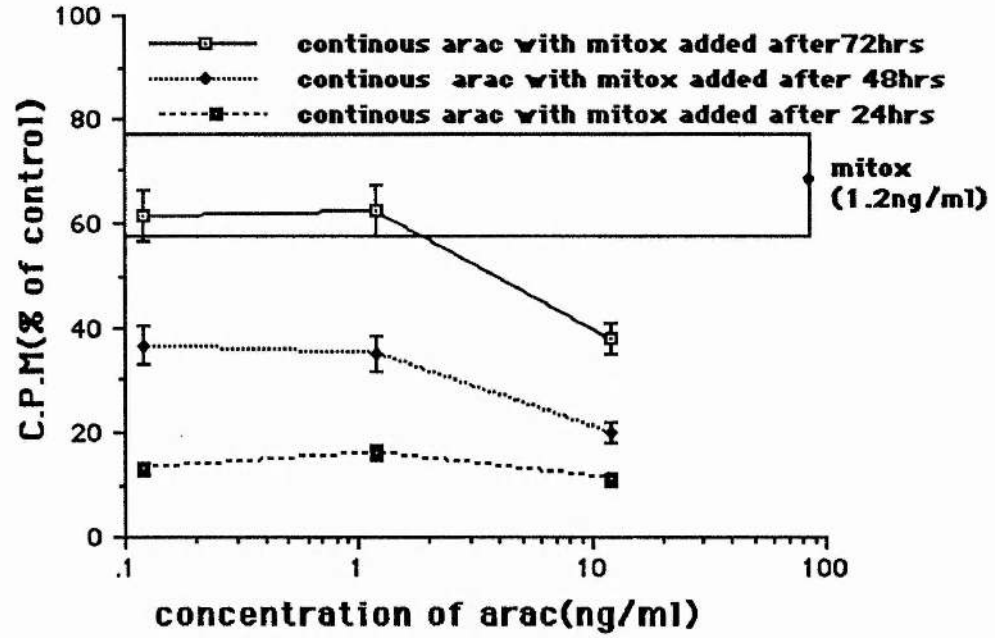


Fig. 49: The effect of exposing NBM cells continously to arac with mitox(1.2ng/ml) added after 24, 48 or 72 hrs later. Cytotoxicity monitored using the (3H)-thymidine uptake assay.

Figure 50 : Flow diagram of 1 hour pulse with mitoxantrone (mitox) alone or in combination with arac followed by addition of arac either immediately after 1-hour pulse with mitoxantrone or 24, 48 or 72 hours later. Cells were labelled with 3HTdR after 96 hours and harvested after 104 hours.

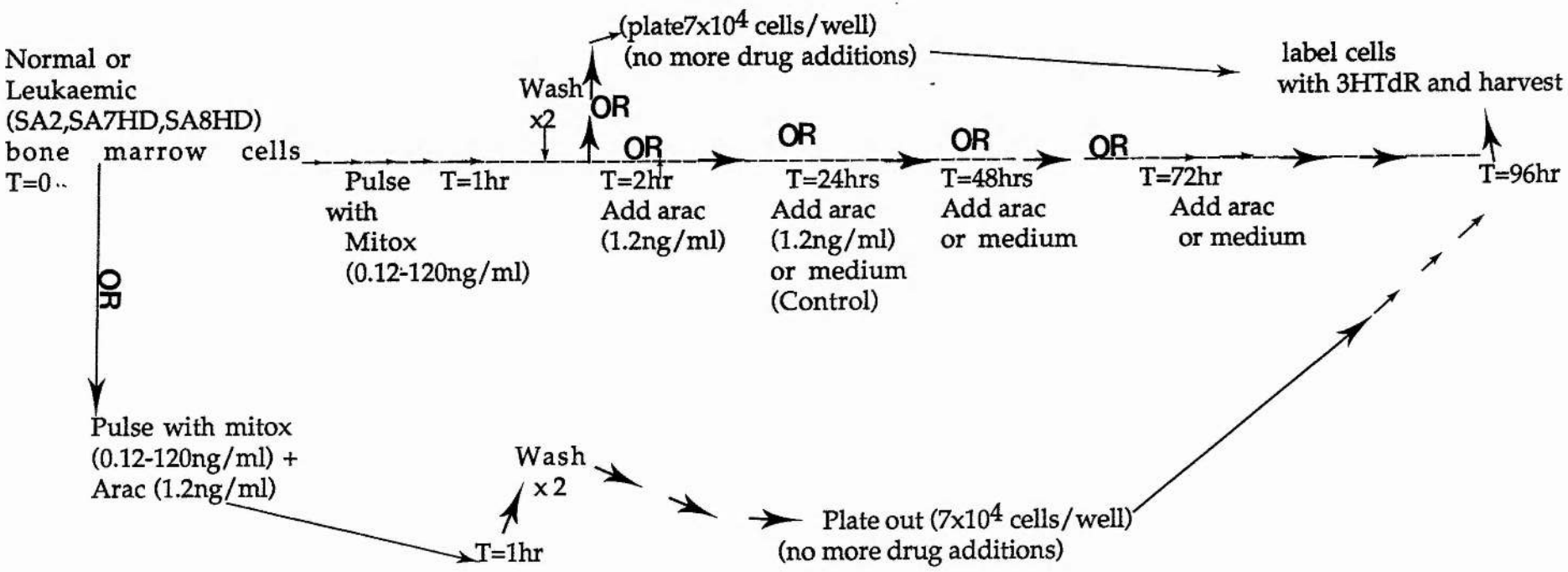


Figure 54 shows the effect of one hour pulse with mitoxantrone on SA7 cell line. As was observed with SA2 cell line, one hour pulse with mitoxantrone (Figure 54) resulted in greater inhibition of DNA synthesis as compared to one hour pulse with Ara-C (Figure 42). In addition, there was not much difference between the effects of one hour pulse and continuous incubation with mitoxantrone on this cell line (compare Figures 33 and 54). When cells of the SA7 cell line were pulsed with mitoxantrone (0.12-120ng/ml) and Ara-C (1.2ng/ml), an antagonistic effect was seen at mitoxantrone concentrations below 120ng/ml (Figure 54). A similar effect was seen when Ara-C was added after 24 hours (Figure 55). Similarly, adding Ara-C 24, 48 or 72 hours following a one hour pulse with mitoxantrone resulted in an increased [3H]TdR uptakes (Figure 58). Thus, whereas inhibition of [3H]TdR uptake following a one hour pulse with Ara-C in the SA7 cell line was schedule dependent, no such effect was observed when the cells were first pulsed with mitoxantrone.

A one hour pulse with mitoxantrone produced less inhibition of DNA synthesis on leukaemic cells from SA8 cell line (Figure 57) as compared to SA7 cell line (Figure 54) particularly using mitoxantrone concentrations below 12ng/ml. Whereas a one hour pulse with both drugs resulted in additive effects using low mitoxantrone concentrations (0.12-1.2ng/ml), a slightly antagonistic effect was seen with 12ng/ml of mitoxantrone (Figure 57). There was no pronounced time-related inhibition of DNA synthesis when Ara-C was added 24, 48 or 72 hours following one hour pulse with mitoxantrone (Figure 58). However, an additive effect was observed when Ara-C was added 72 hours rather than 48 hours after one hour pulse with mitoxantrone. With the 48-hour schedule, increased [3H]TdR uptake above that seen with one hour pulse with mitoxantrone alone was observed. In addition, greater inhibition

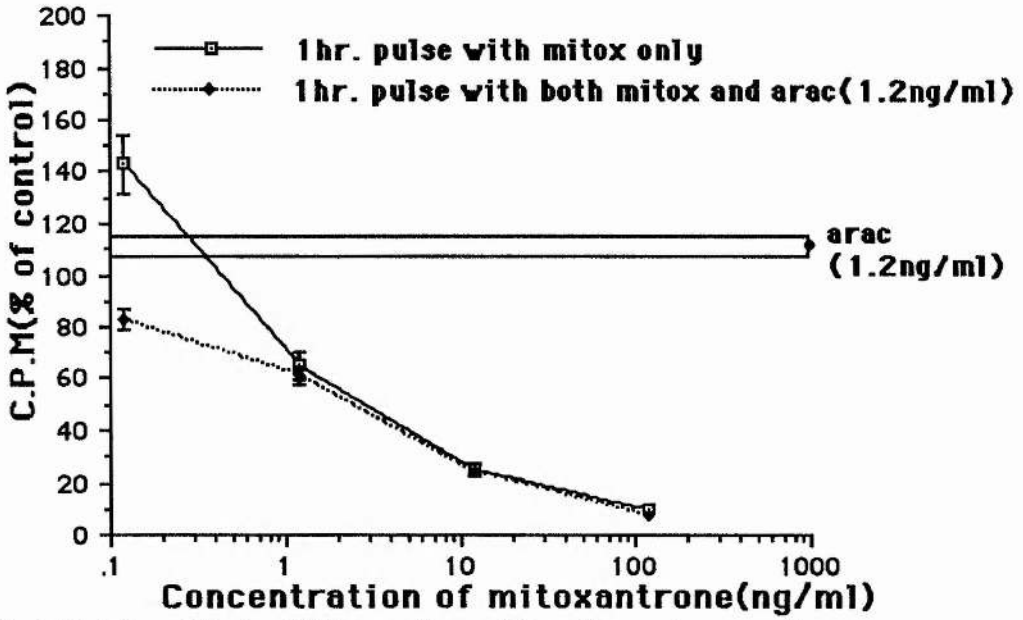


Fig.51: The effect of 1hr. pulse with mitoxantrone alone or in combination with arac on SA2 cell line. Cytotoxicity was determined using the (3H)-thymidine uptake assay.

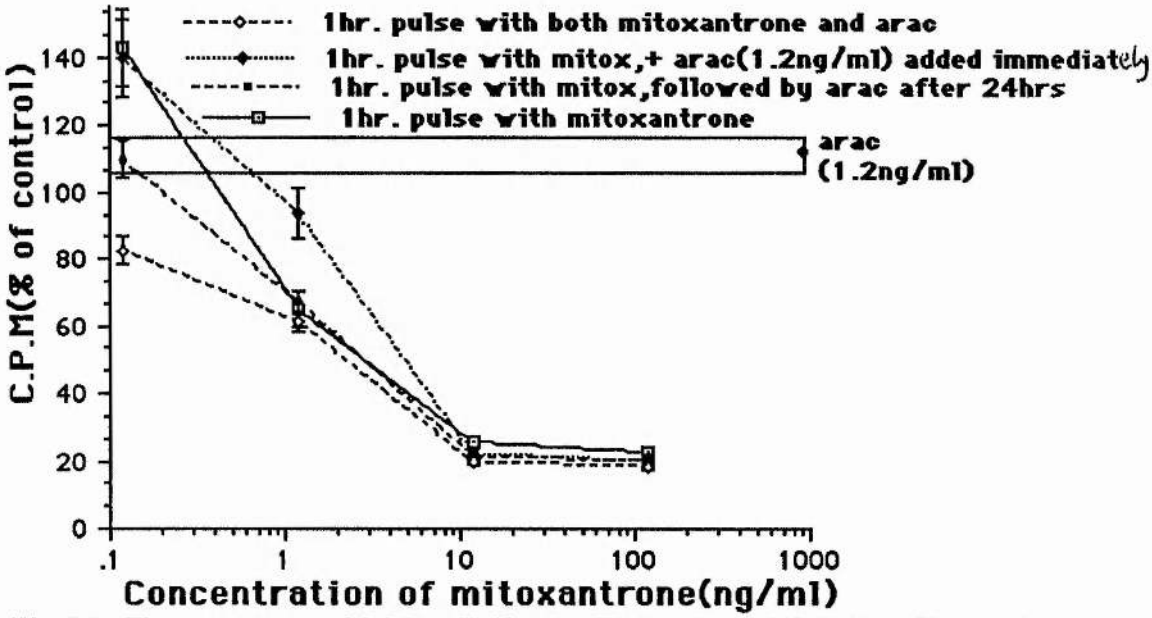


Fig.52: The response of SA2 cell line to 1hr. pulse with both mitox and arac; or, 1hr pulse with mitox followed by arac(1.2ng/ml) either immediately or 24, 48, or 72hrs later. Cytotoxicity was determined using (3H)-thymidine uptake assay.

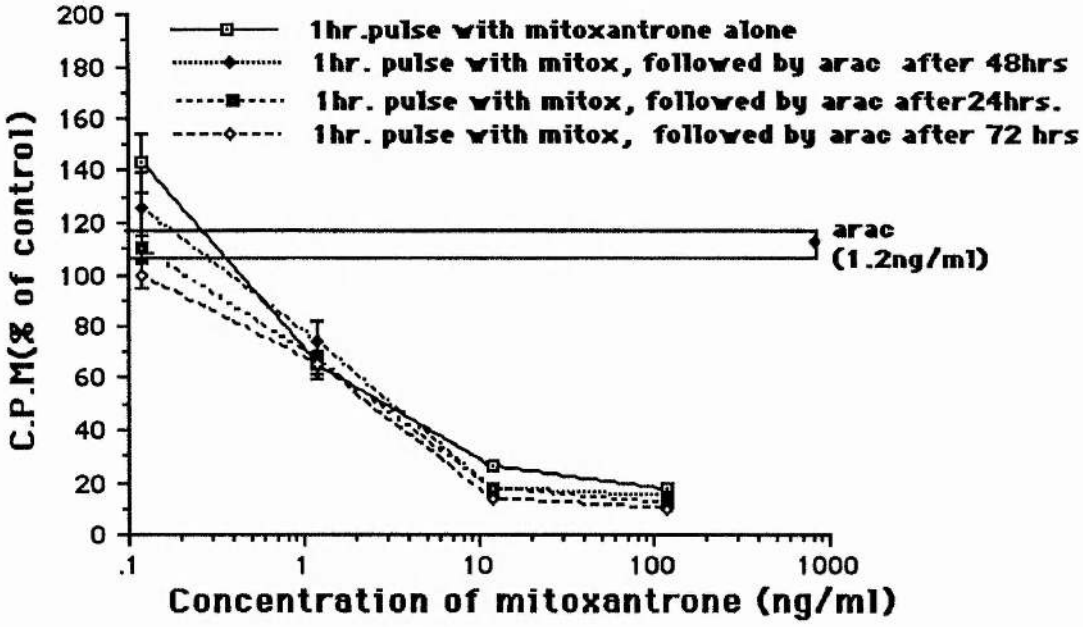


Fig.53: The response of SA2 cell line to 1hr. pulse with mitoxantrone alone or with arac(1.2ng/ml) added after 24,48,or 72hrs.

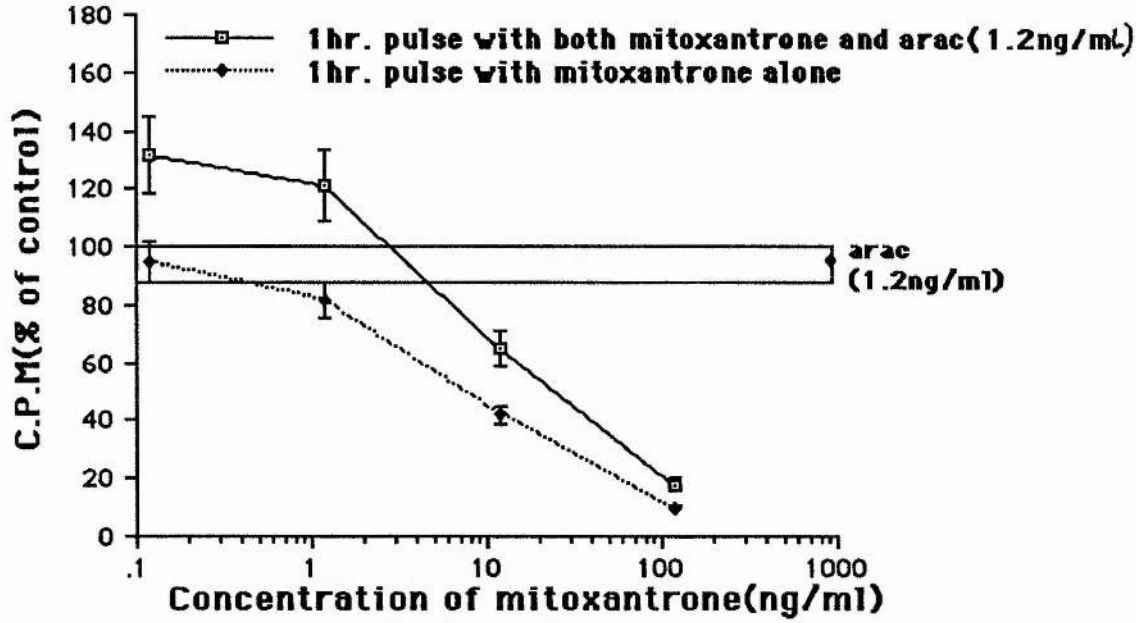


Fig.54: The effect of 1hr. pulse with mitoxantrone alone or in combination with arac on SA7 cell line.
Cytotoxicity was determined using the (3H)-thymidine uptake assay.

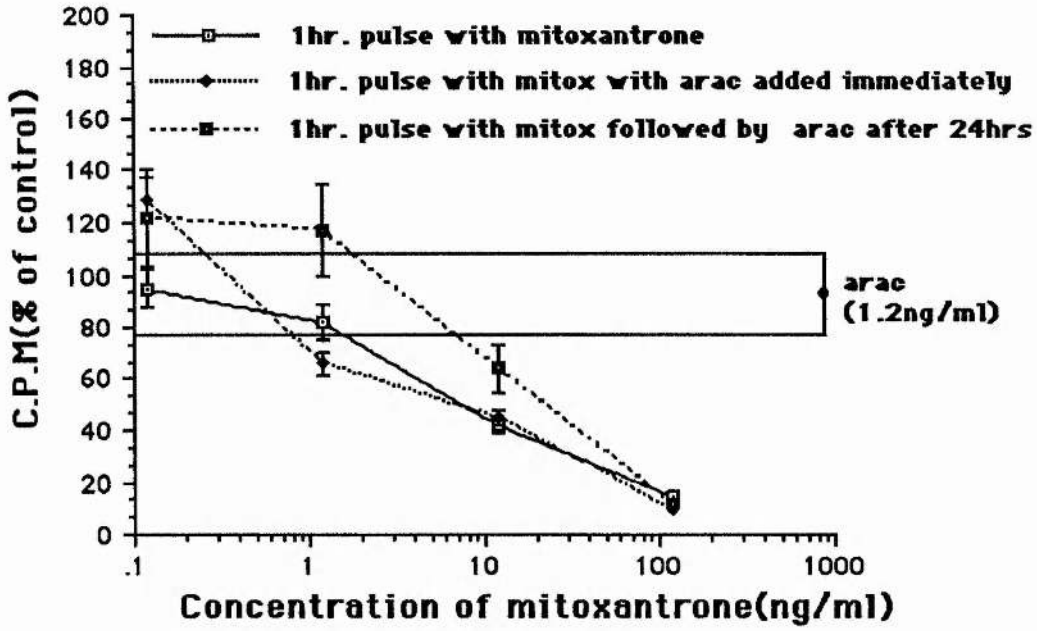


Fig.55: The response of SA7 cell line to 1hr. pulse with mitoxantrone either alone or followed immediately or after 24hrs with arac(1.2ng/ml) Cytotoxicty was determined using the (3H)-thymidine uptake assay.

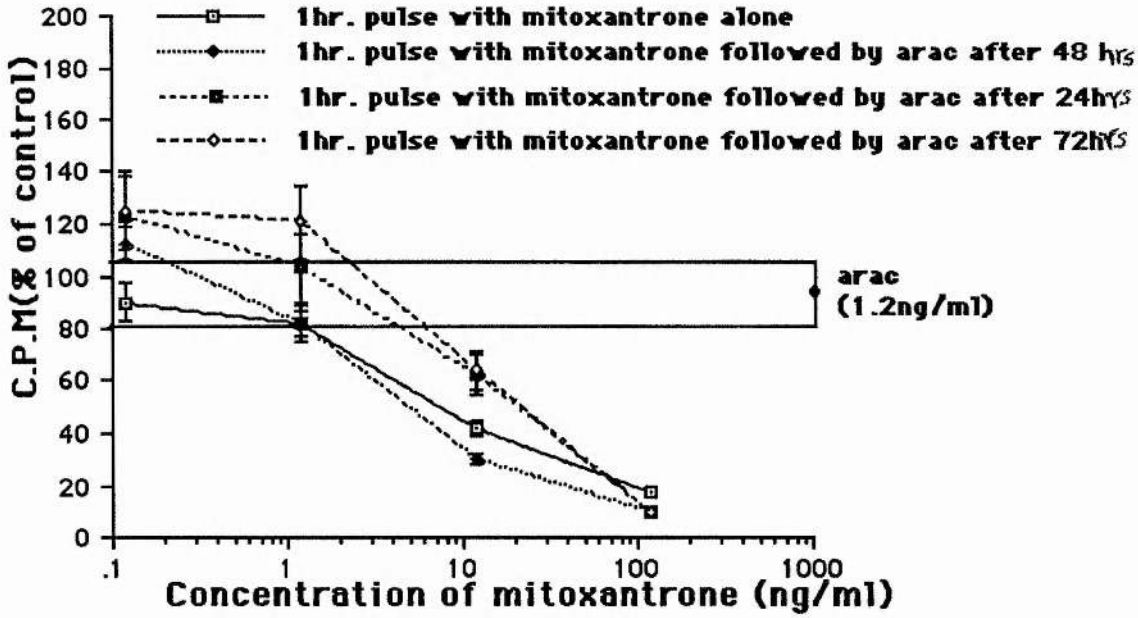


Fig.56: The response of SA7 cell line to 1hr pulse with mitoxantrone alone or followed by arac (1.2ng/ml) 24,48 or72 hrs later. Cytotoxicity was determined using the (3H)-thymidine uptake assay.

of DNA synthesis was observed if Ara-C was added immediately after pulsing the cells with mitoxantrone as compared to when Ara-C was added 24 hours later (Figure 59).

This effect was more pronounced using a low mitoxantrone concentration (0.12-1.2ng/ml). The SA8 cell line therefore showed no schedule dependent effects following a one hour pulse with either Ara-C or mitoxantrone.

As was observed with leukaemic cell lines SA2, SA7HD and SA8HD, a one hour pulse with mitoxantrone resulted in greater inhibition of DNA synthesis in normal bone marrow cells (Figure 60) as compared to similar treatment with Ara-C (Figure 47). No additive effect was observed when Ara-C was added 24, 48 or 72 hours following one hour pulse of normal bone marrow cells with mitoxantrone. Similarly, pulsing the cells with both drugs for one hour did not make any difference as compared to when mitoxantrone alone was used (Figure 61). Increased [3H]TdR uptake was observed if Ara-C was added either immediately following one hour pulse with mitoxantrone or 24 hours later (Figure 62). The effect was more pronounced with the latter (24 hours) schedule.

3.3 Autoradiography: this technique was employed to determine the in vitro labelling indices of the leukaemic cell lines SA2, SA7 and SA8 and also the effect of continous incubation with mitoxantrone on the labelling indices.

3.3.1 Experimental Procedure: A single cell suspension was prepared from the following leukaemic cell lines (SA2, SA7HD and SA8HD) and plated at 7×10^4 cells per well in a microtitre plate. WEHi conditioned medium was added to give final concentration of 10% (except SA2 leukaemic cell line). Mitoxantrone (0.12-120ng/ml) was then added and

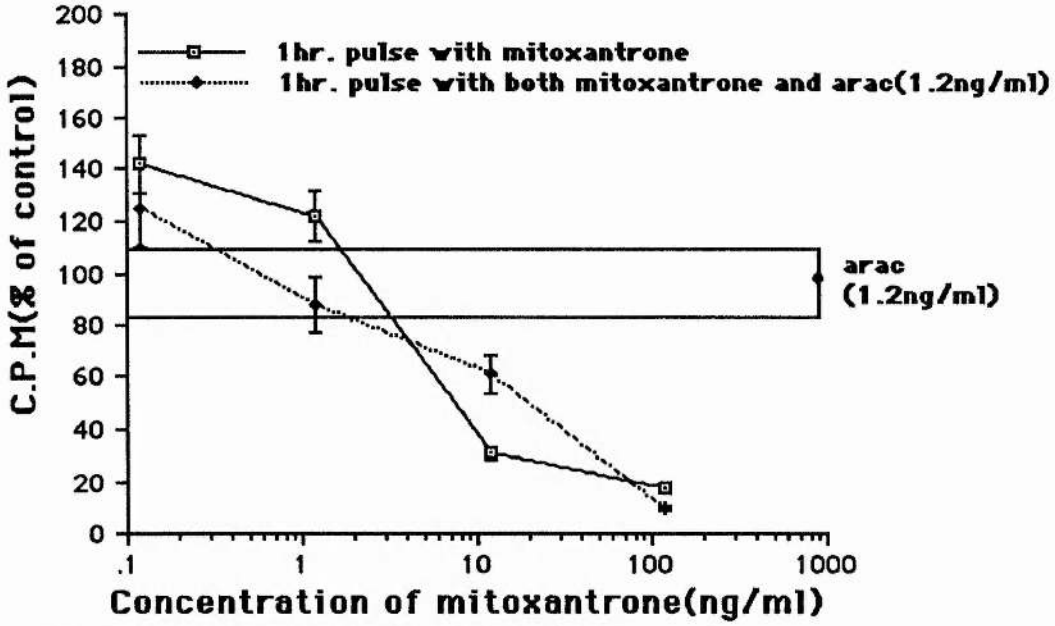


Fig.57: The response of SA8 cell line to 1hr. pulse with mitoxantrone alone or in combination with arac. Cytotoxicity was determined using the (3H)-thymidine uptake assay.

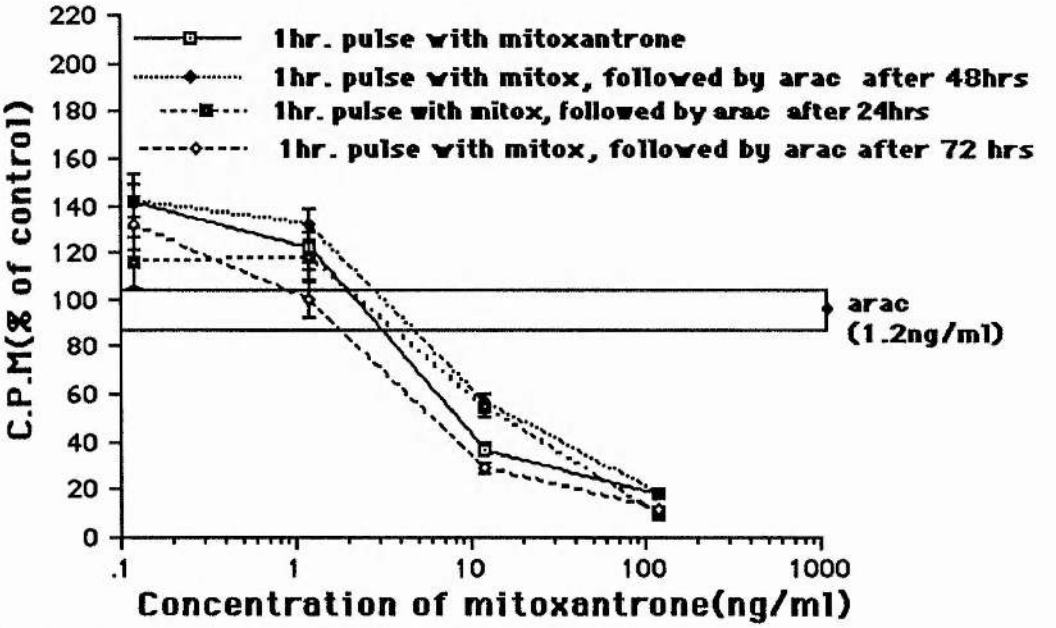


Fig.58: The response of SA8 cell line to 1hr. pulse with mitoxantrone alone; or with arac(1.2ng/ml), added either 24, 48, or 72 hrs later. Cytotoxicity was determined using the (3H)-thymidine uptake assay.

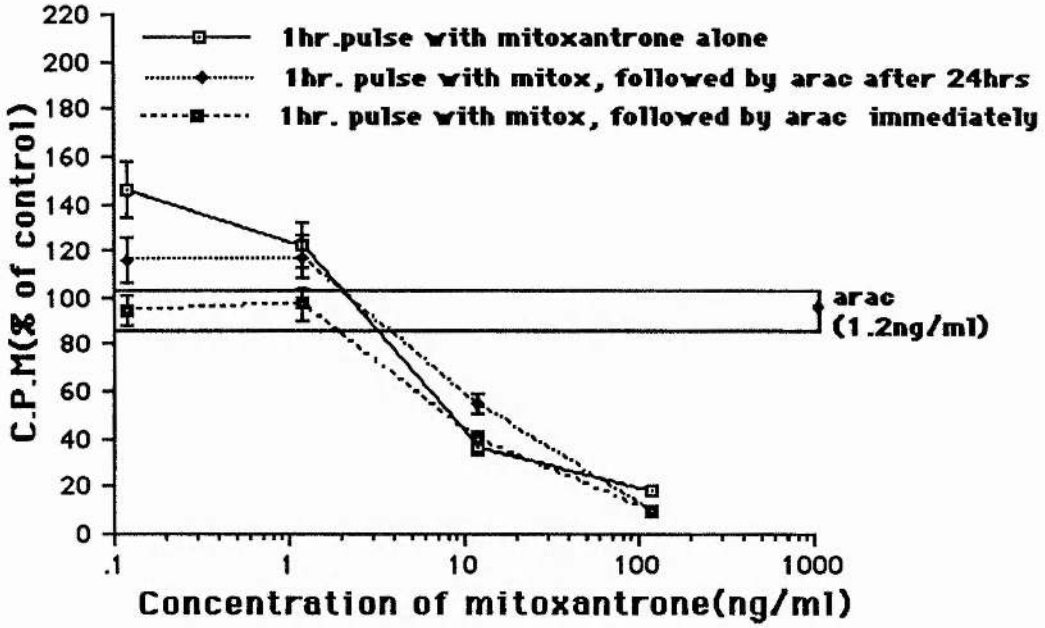


Fig.59: The response of SA8 cell line to 1hr. pulse with mitoxantrone alone or followed by arac(1.2ng/ml) either immediately or 24hrs later. Cytotoxicity was determined using the (3H)-thymidine uptake assay

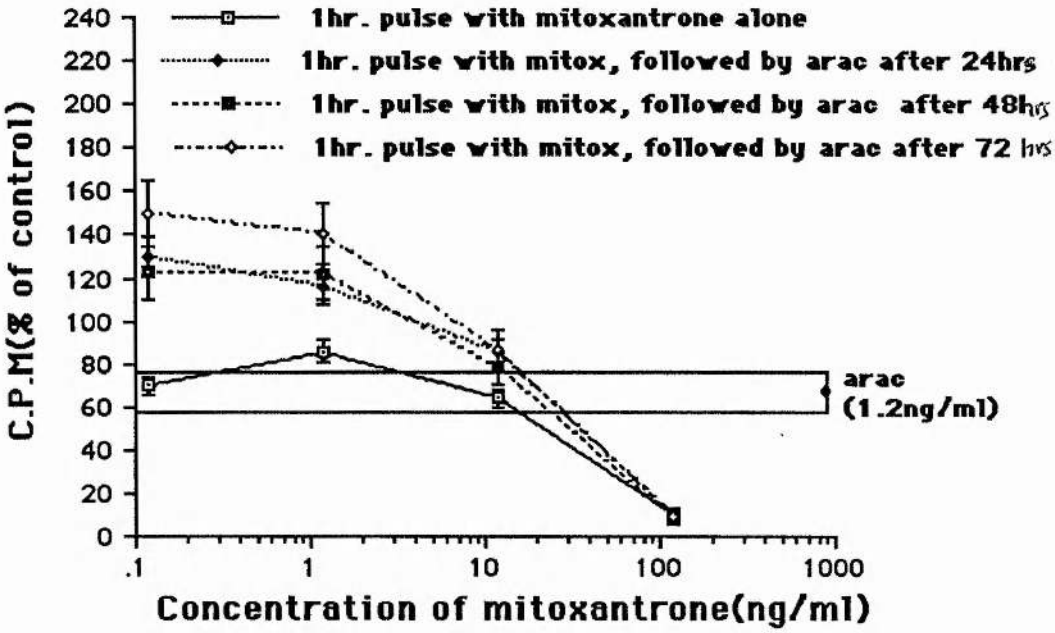


Fig.60: The response of NBM cells to 1hr. pulse with mitoxantrone alone; or with arac(1.2ng/ml) added after 24, 48, or 72hrs . Cytotoxicity was determined using the (3H)-thymidine uptake assay.

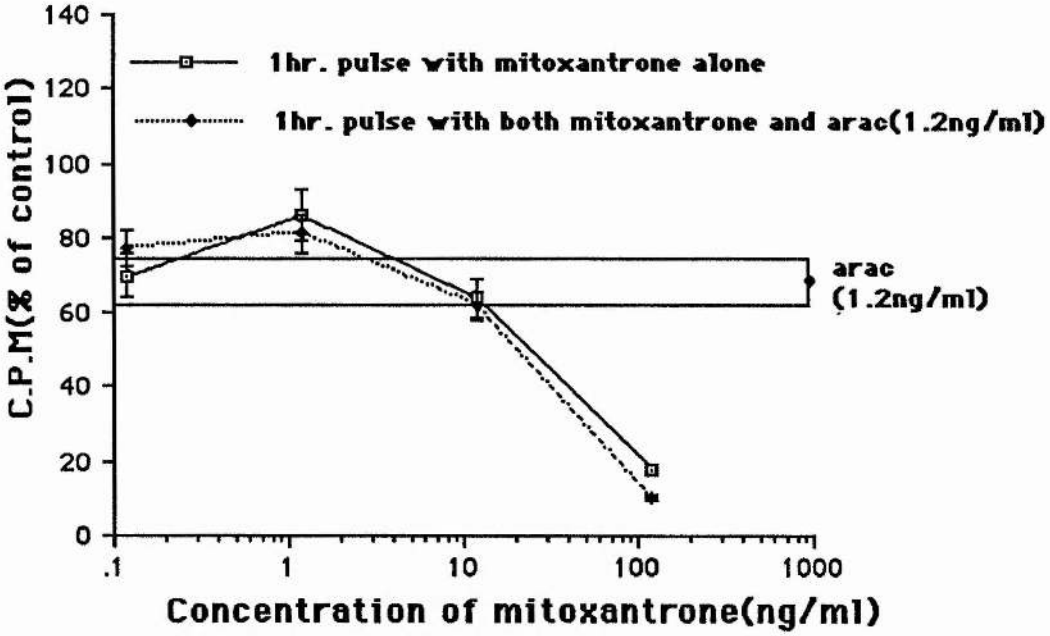


Fig.61: The response of NBM cells to 1hr. pulse with mitoxantrone alone or in combination with arac.

Cytotoxicity was determined using the (3H)-thymidine uptake assay.

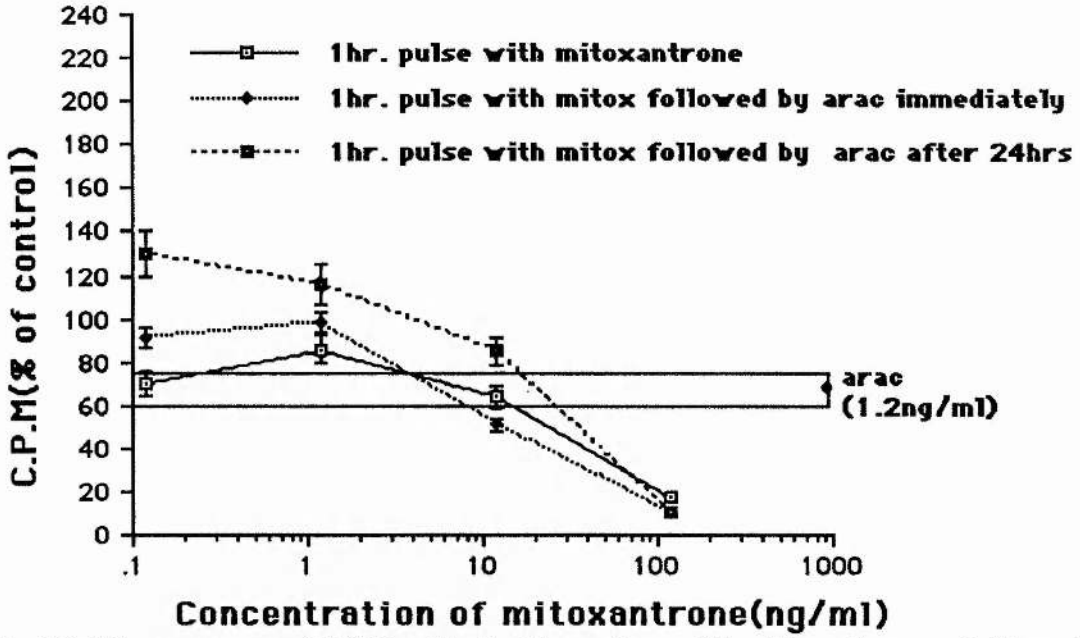


Fig.62: The response of NBM cells to 1hr. pulse with mitox alone or followed by the addition of arac(1.2ng/ml) either immediately or 24 hrs later.

the plates incubated. With the SA7 cell line, cells were pulsed with [3H]TdR for one hour from days 1 - 4 and washed and then cytocentrifuged onto ethanol-washed slides. With SA2 and SA8HD, the plates were incubated for four days. On the fourth day, the cells were pulsed with [3H]TdR for one hour and slides prepared as described for SA7 cell line. All slides were processed and developed (section 2.2.4.2) and the labelling indices were obtained by counting the number of cells that incorporate the label and expressing this as a percentage of the whole cell population.

Results: Figure 63 shows the change in labelling index of both untreated and mitoxantrone treated cells of the SA7 leukaemic cell line with duration of culture. The labelling index began to rise (in untreated cells) from day one almost exponentially and reaches its peak on day three and fell a little on day four. With the cells continuously exposed to mitoxantrone (120ng/ml) the labelling index was depressed to 3% of

control on day one and by days two through to four, it was virtually zero. This is in complete agreement with the result of other cytotoxicity assays using this concentration of mitoxantrone. Virtually 100% inhibition of [3H]TdR uptake was seen at this concentration (Figure 54) and similar effects were seen using DiSC assay and cell number assessment methods (Figure 5). The SA7 had a labelling index of 29%, while the SA2(plate 2) and SA8(plate 3) had labelling indices of 37 and 28% respectively after four days in culture. Qualitatively similar dose-response curves were obtained on the effect of mitoxantrone on SA2 (Figure 64) and SA8 cell lines (Figure 65) using autoradiography or [3H]TdR uptake assay. Quantitatively however, the labelling index method seemed to underestimate cytotoxicity produced by low concentrations of mitoxantrone (0.12-1.2ng/ml).

Plate 1: A mixture of live and dead SA7HD leukaemic bone marrow cells. Dead cells(DC) were stained dark-green by fastgreen-nigrosin while live cells(LC) assume normal Jenner-Giemsa counterstain(Magnification x 1300).

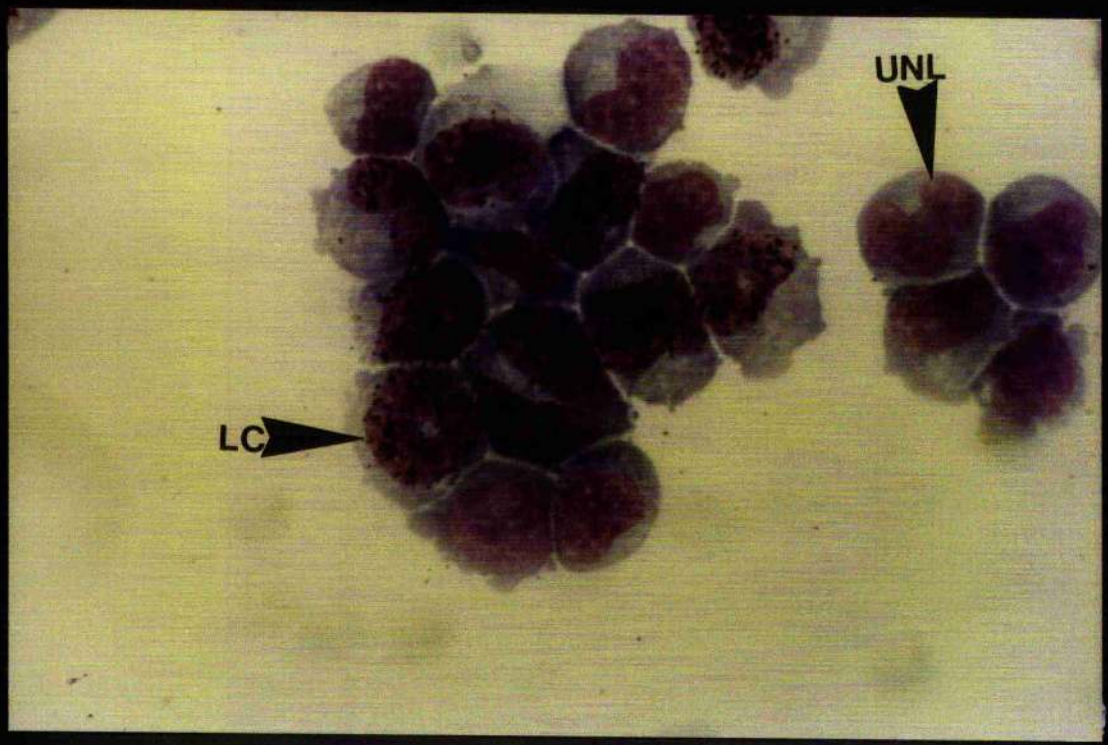


Plate 2: An autoradiograph of leukaemic bone marrow cells from SA2 leukaemic cell line. The cells were pulsed with (3H)-thymidine following 4-days incubation. Cells synthesizing DNA incorporated the label while those cells that were not in S-phase remained unlabelled .
LC= Labelled cells; UNL =unlabelled cells(magnification X1300)

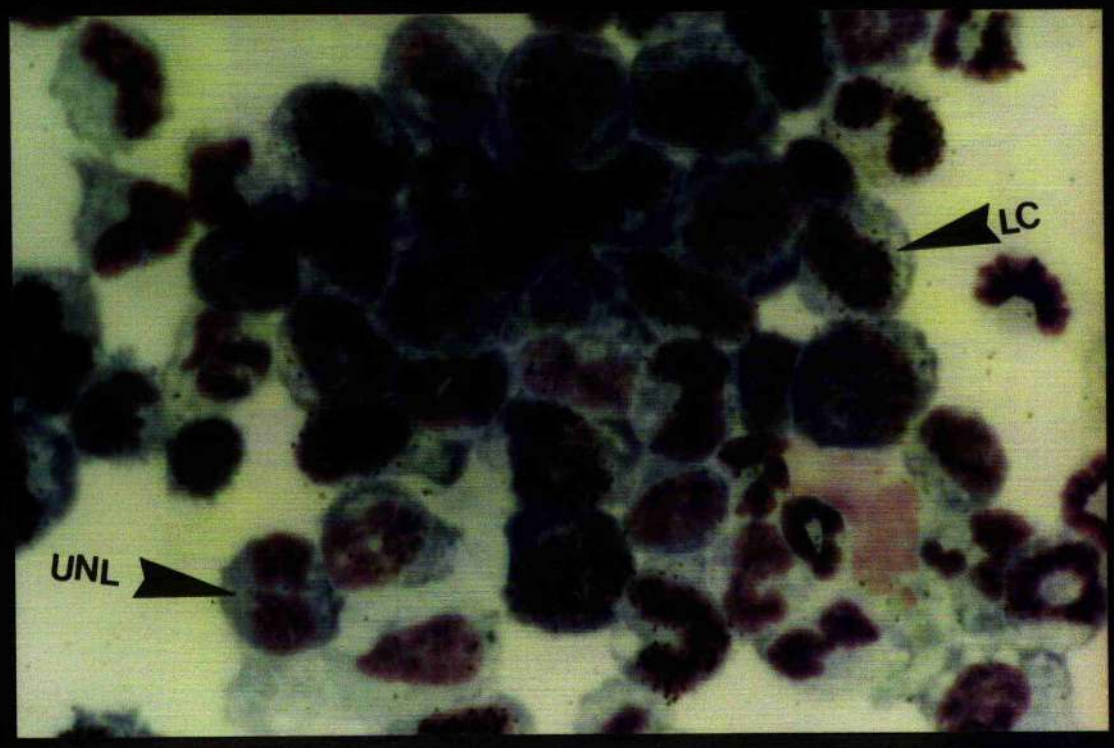


Plate 3: An autoradiograph of SA8 high cell dose passage(SA8HD) leukaemic bone marrow cells. The cells were pulsed with (3H)-thymidine following 4- days incubation in culture. Cells undergoing DNA synthesis were labelled(LC) whereas those cells that were not in S- phase remained unlabelled(UNL)(Magnification X 1300).

Plate 4: Spleen cells of a mouse bearing SA7HD leukaemia that was treated <u>in vivo</u> with 2 doses of mitoxantrone(3mg/Kg). No apparent evidence of leukaemic infiltration of spleen is seen. Most of the cells are normal lymphocytes(NL) with the occasional blast cell(BL). Compare this with plate 6 (Magnification X 1300).

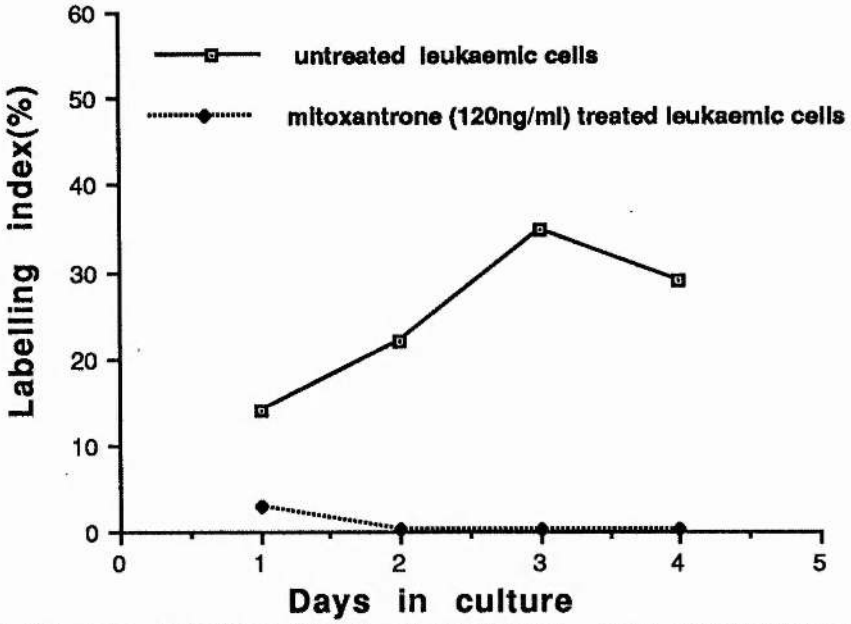


Fig.63: The labelling index of untreated and mitoxantrone treated leukaemic cells from the SA7 cell line determined using autoradiography.

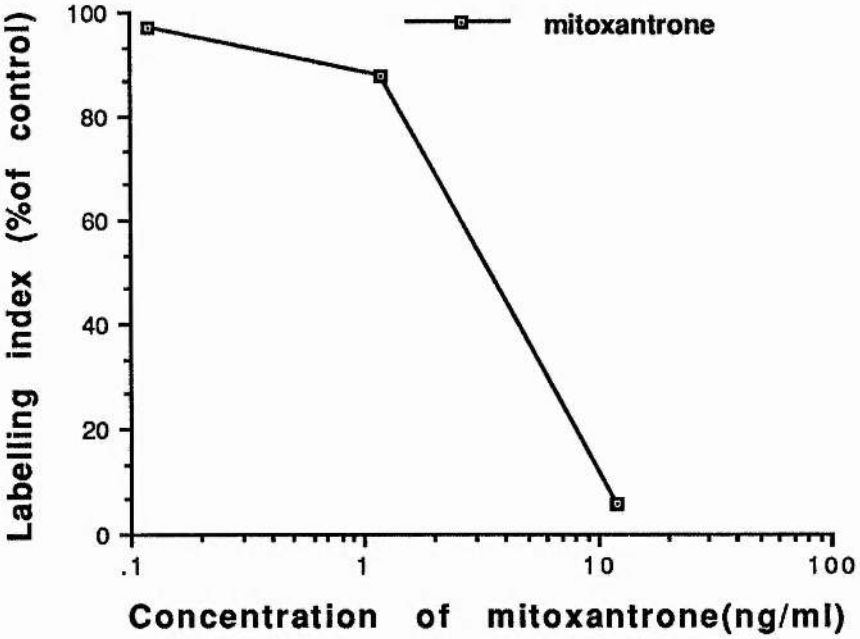


Fig.64: The effect of mitoxantrone on in vitro labelling index of SA8 cell line monitored using autoradiography.

3.4 Granulocyte-Macrophage Colony Forming Cell Assay: the effects of Ara-C and mitoxantrone alone or in combination on normal bone

marrow progenitor cells were determined using granulocyte-macrophage colony forming cell (GM-CFC) assay.

Experimental Procedure: Various incubation schedules including one hour pulse and continuous incubation methods were employed. In the one hour pulse method, normal bone marrow cells were pulsed with Ara-C (0.12-120ng/ml) for one hour and plated in agar with the cells (5 x 10^4) suspended in either plain medium or medium containing mitoxantrone (0.27ng/ml). Alternatively the NBM cells were pulsed with mitoxantrone (0.012-120ng/ml) for one hour and plated in agar with the cells suspended in either plain medium or medium containing Ara-C (22ng/ml). In contrast, in the continuous incubation method, 5 x 10^4 NBM cells were plated out in agar while suspended in medium containing Ara-C or mitoxantrone (0.012-120ng/ml). Colonies were scored on day 7 blindly or by an independent observer. Dose-response curves were obtained by plotting the number of colonies in drug treated samples expressed as a percentage of control colonies as a function of drug concentration.

Results: Figure 66 shows the dose-response curve of normal (murine) bone marrow myeloid progenitor cells to Ara-C and mitoxantrone treatment In vitro using the continuous incubation method. It is apparent that mitoxantrone seems dose for dose more toxic than Ara-C on progenitor cells in vitro. When bone marrow cells were pulsed with Ara-C (120ng/ml) for one hour, no cytotoxic effect was observed. However, if mitoxantrone (36ng/ml) was added and the cells pulsed with both drugs for one hour, an additive cytotoxic effect was observed (Figure 67). Similar additive effects were observed when the cells were pulsed with Ara-C for one hour and then continuously exposed to mitoxantrone (0.27ng/ml) for the duration of the assay (Figure 68). Furthermore, one hour pulse with mitoxantrone followed by

continuous incubation with Ara-C (22ng/ml) resulted in additive cytotoxic effect (Figure 68) as compared to one hour pulse with mitoxantrone alone (Figure 69). Using the latter schedule, one hour pulse with 120ng/ml of mitoxantrone resulted in inhibition of all colony growth. This is in complete agreement with the result obtained using [3H]TdR uptake assay (Figure 62) showing that [3H]TdR uptake was inhibited by nearly 100% using similar schedule and concentration of mitoxantrone.

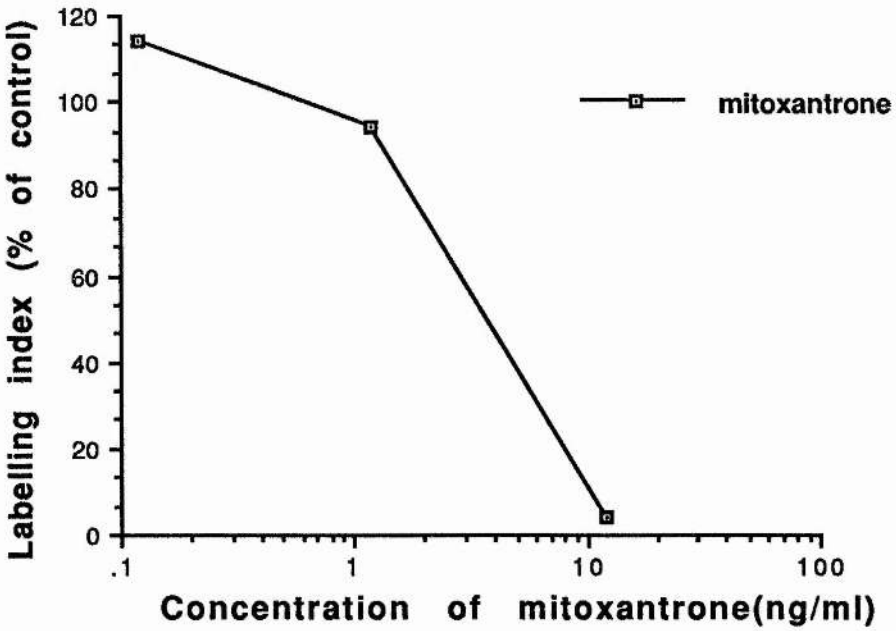


Fig. 65:The effect of mitoxantrone on the in vitro labelling index of the SA8 leukaemic cell line monitored using autoradiography.

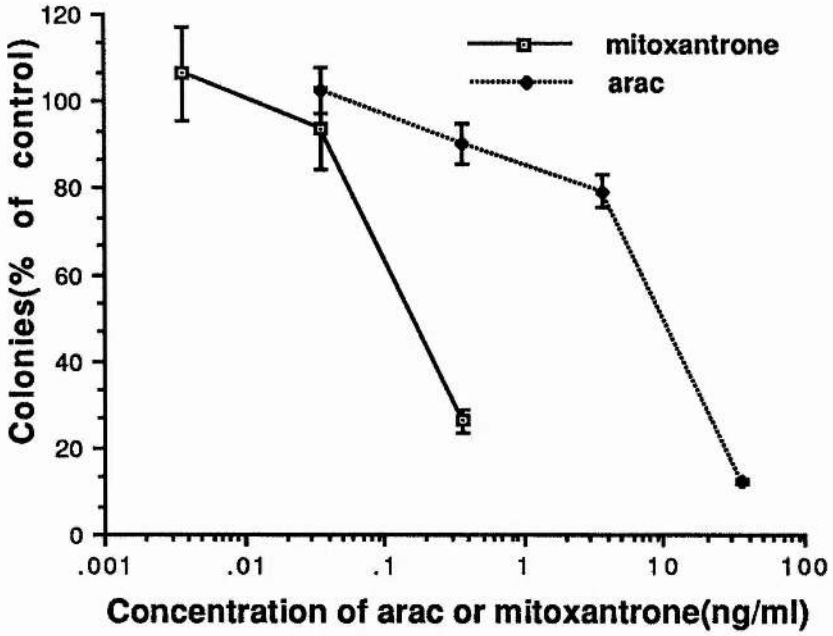


Fig. 66: The response of normal myeloid progenitor cells to mitoxantrone or arac treatment in vitro monitored using the GM-CFC assay.

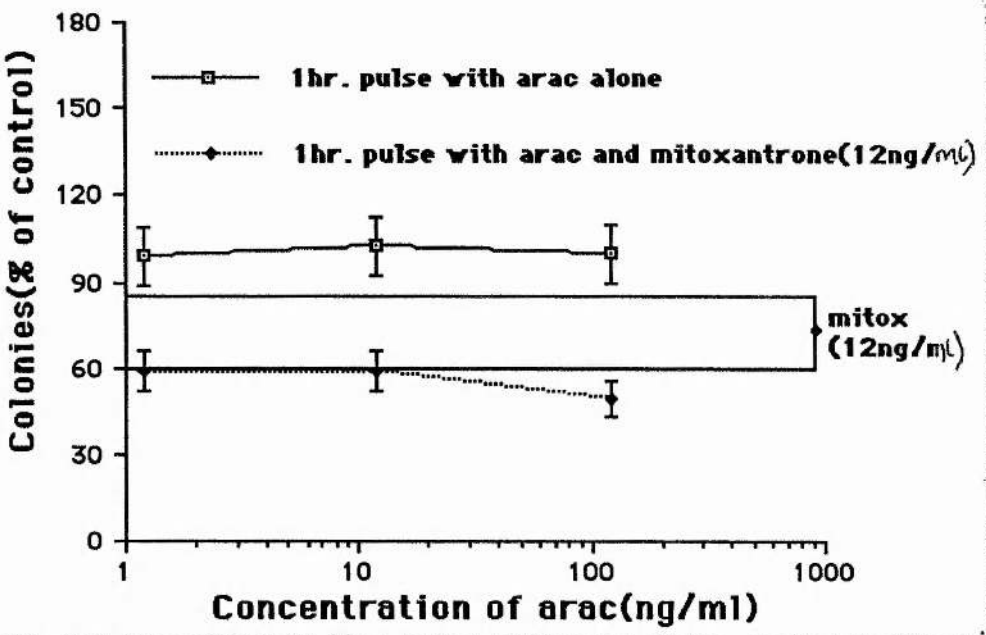


Fig.67: The effect of 1hr. pulse with arac alone or in combination with mitoxantrone on normal bone marrow myeloid progenitors in vitro, monitored using the GM-CFC assay.

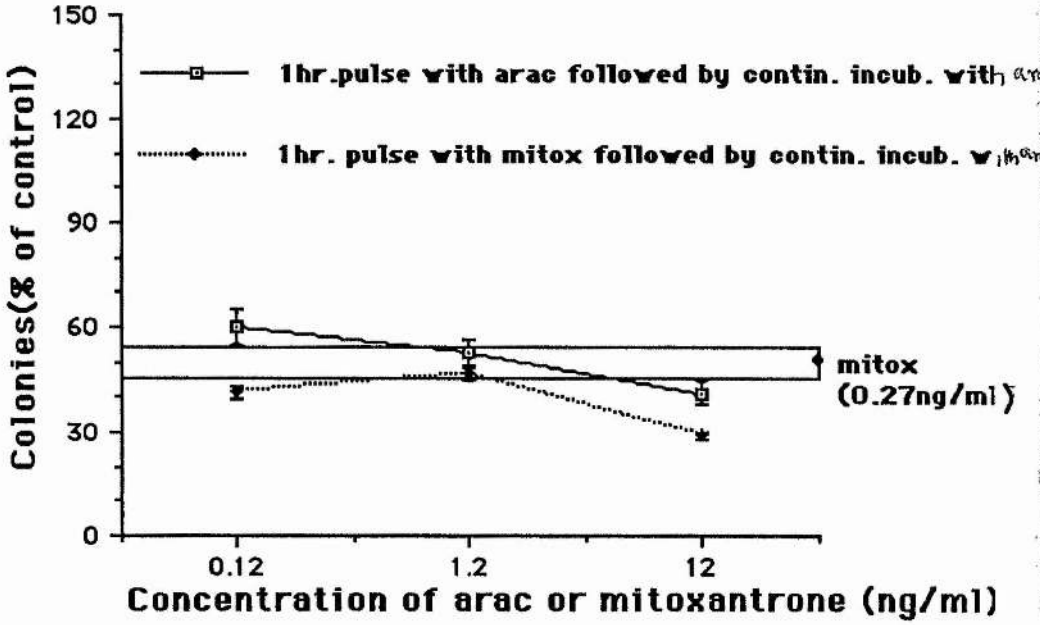


Fig.68: The response of GM-CFCS to 1hr. pulse with arac or mitoxantrone followed by contin. incub. with mitox(0.27ng/ml) or arac(22ng/ml) respectively

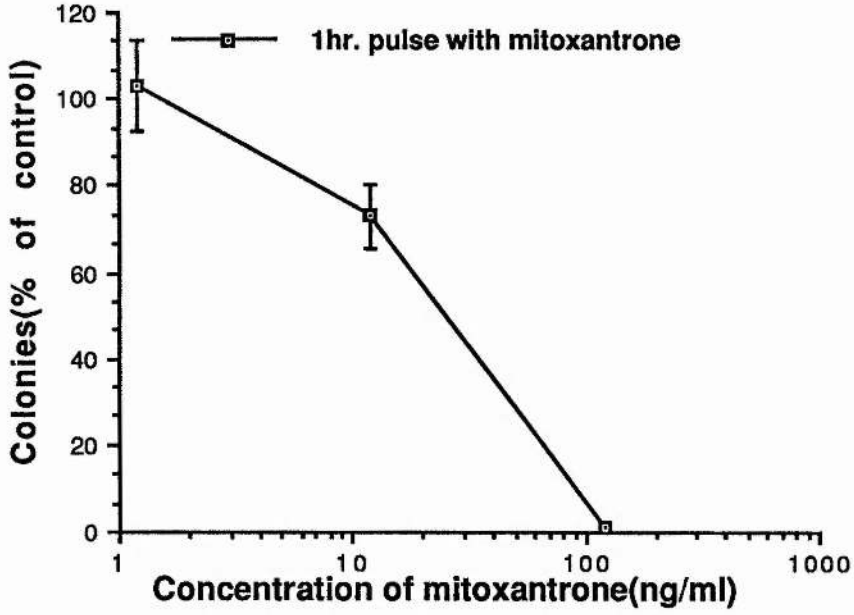


Fig.69: The effect of 1hr. pulse with mitoxantrone on myeloid progenitor cells monitored using GM-CFC assay.

4. RESULTS

CHAPTER FOUR

4. Growth Factor Responses of Leukaemic Cell Lines

In this chapter, the growth factor-induced proliferative responses of a primary leukaemic cell line (81287) and its passaged progeny (81287T1,81287T6 and 81287T10) as well as those of SA2, SA7 low cell dose (SA7FT5), SA8 low cell dose transplant (SA8FT14) and SA8 high cell dose transplant (SA8HD) cell lines were monitored using the [3H]TdR uptake assay.

4.1 Experimental Procedure: A single bone marrow or spleen cell suspension was prepared from the following leukaemic cell lines (81287, SA7FT5 and SA8HD) and plated at 7×10^4 cells per well in a microtitre plate. 100µl each of WEHi conditioned medium, L929 conditioned medium or combinations of the two to give final concentrations of 5, 10 or 20% in each well was added. Control wells received only cells and medium i.e no growth factors were added. After 4 days incubation, the cells were labelled with [3H]TdR, harvested and the amount of radioactive precursor incorporated by the cells was determined (section 2.2.2). Growth factor dose-response curves were prepared by plotting the amount of radioactivity incorporated by the cells (as counts per minute, c.p.m.) as a function of growth factor concentration.

Results: The responses of spleen cells (Figure 70) and bone marrow cells (Figure 71) of the primary leukaemic cell line 81287 to WEHi alone or in combination with L929 conditioned medium are shown. This cell line was not very responsive to both WEHi, L929 and their combination. Whereas additive effects were seen with combination of 10% WEHi and 10% L929 with spleen cells, there was no difference in the response of bone marrow cells to the same concentrations. With first passage in

syngeneic mice (81287TI), bone marrow response to the growth factors improved slightly (Figure 72). Additive effects were seen with 5% WEHi and 5% L929 and also with 20% WEHi and 20% L929.

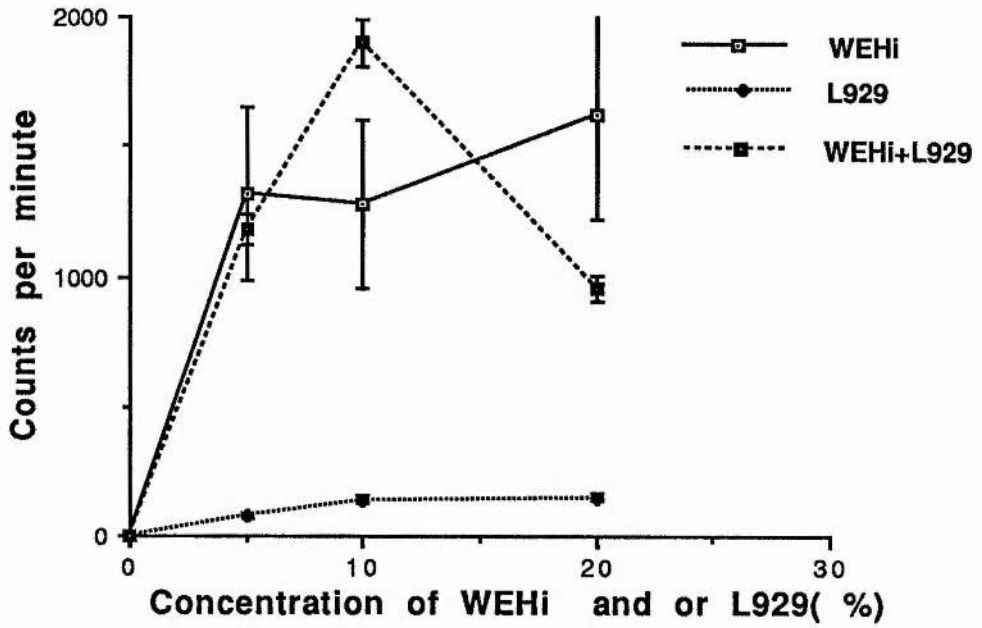


Fig. 70: The response of spleen cells from leukaemic cell line 81287 to WEHi, L929, or combinations of the two conditoned media monitored using (3H)-thymidine uptake assay.

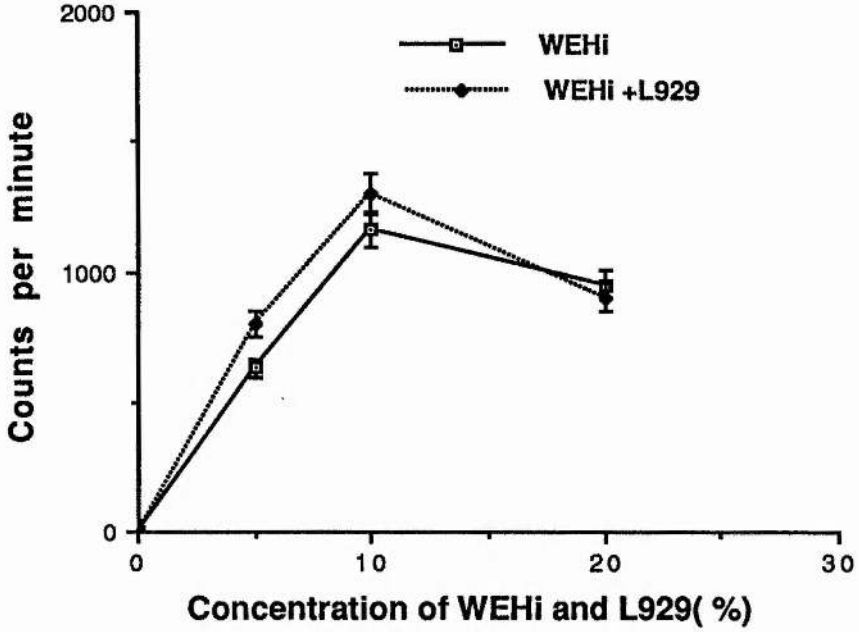


Fig.71:The response of bone marrow cells from the 81287 cell line to WEHi alone or in combination with L929 monitored using (3H)-thymidine uptake assay.

Even after the tenth passage, there was only a modest improvement in growth factor responsiveness and an additive effect was observed with 10% WEHi and 10% L929 (Figure 73).

The responses of spleen cells (Figure 74) and bone marrow cells (Figure 75) of the low cell dose transplant cell line SA7FT5 to WEHi alone, L929 alone or their combinations are shown. Both spleen and bone marrow cells seem equally sensitive to WEHi although spleen cells were a little bit more responsive. However, both spleen and bone marrow cells were particularly insensitive to L929 conditioned medium. All combinations of WEHi and L929 studied resulted in an additive proliferative effect in spleen cells but in bone marrow cells only 10% WEHi and 10% L929 resulted in a slight additive effect. Thus both 81287 (even after 10 passages) and low cell dose transplant leukaemic cell lines were not very responsive to growth factors in vitro. In contrast, the SA8 high cell dose transplant cell line was very responsive to WEHi conditioned medium

alone, L929 conditioned medium alone and increased proliferative response was observed with combinations of the two conditioned media (Fig.76).

4.2 Effects of Foetal Calf Serum and Transferrin on the In Vitro Proliferative Response of SA2 Leukaemic Cell Line: The SA2 leukaemic cell line is unique in the sense that it was capable of in vitro growth even if no growth factor(s) was added to the cells in culture. The effects of foetal calf serum and transferrin on the proliferative response of the leukaemic cell line were investigated.

4.2.1 Experimental Procedure: A single cell suspension was prepared from the femora of mice bearing the SA2 leukaemic cell line (section 2.1.3). The cells were suspended in medium with or without foetal calf serum (10%) and 7×10^4 cells were plated per well in a microtitre plate. Graded concentrations of WEHi conditioned medium, L929 conditioned medium or combinations of the two conditioned media were added to give final concentrations of 5, 10 or 20% in each well. In a separate experiment, the cells were suspended in serum free medium and transferrin with or without growth factors were added. The transferrin was either saturated with iron or else used unsaturated at the concentration of 10μg/ml. Since there may well be up to 1% serum in the growth factor conditioned media used, an equivalent amount of foetal calf serum (1%) was added to the control wells that received no growth factors.

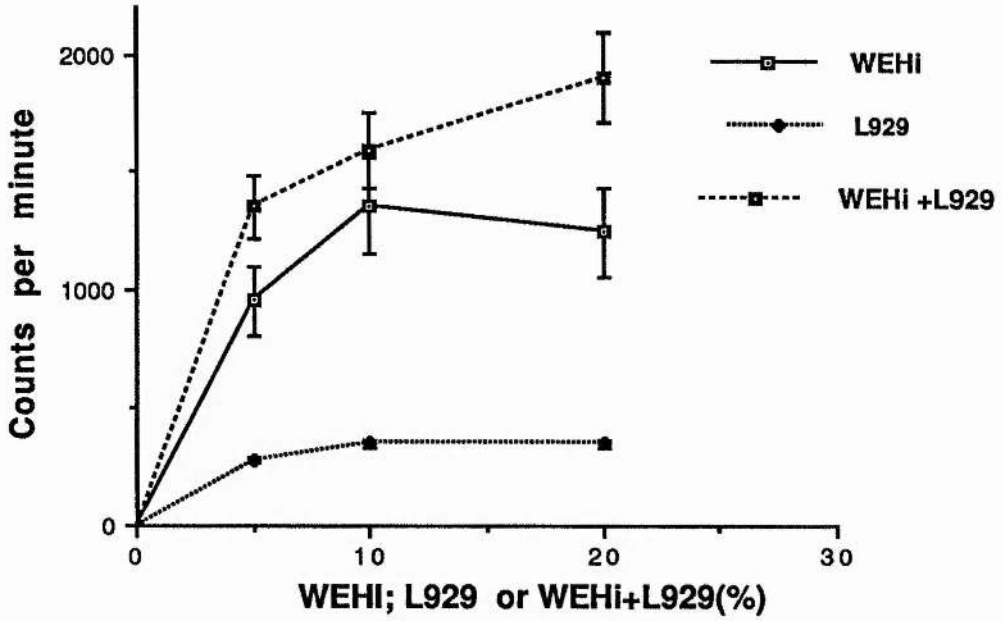


Fig.72: The response of bone marrow cells from 81287T1 cell line to growth factors monitored in vitro using the (3H)-thymidine uptake assay.

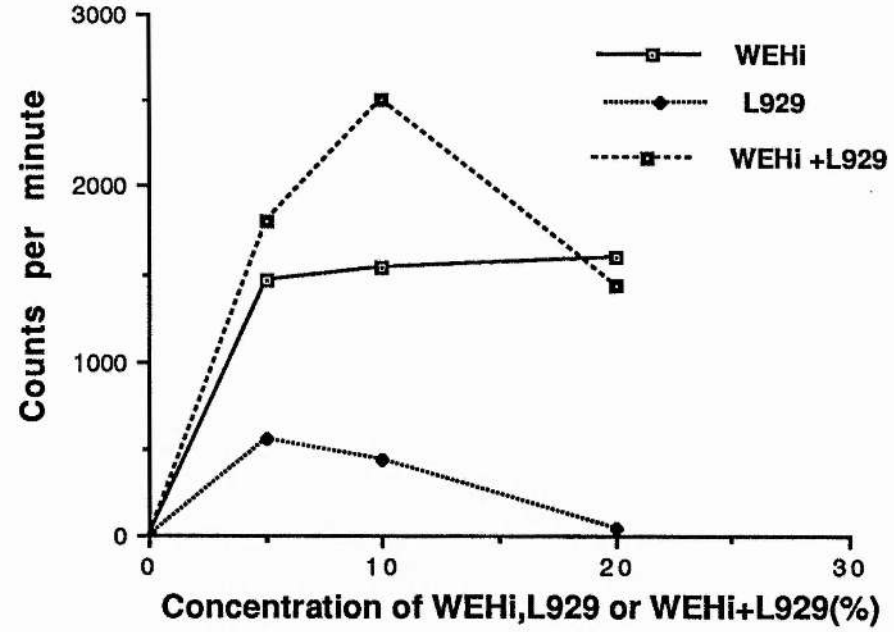


Fig.73: The response B.M cells of 81287T10 cell line to growth factors monitored in vitro using the (3H)-thymidine uptake assay.

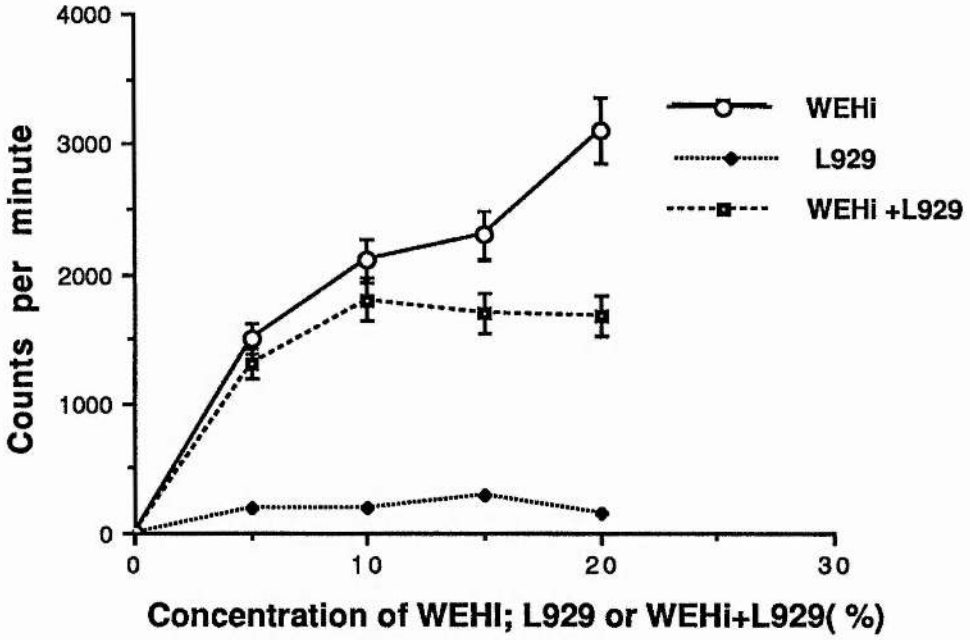


Fig.74: The response of spleen cells from SA7FT5 cell line to growth factors monitored in vitro using (3H)-thymidine uptake assay.

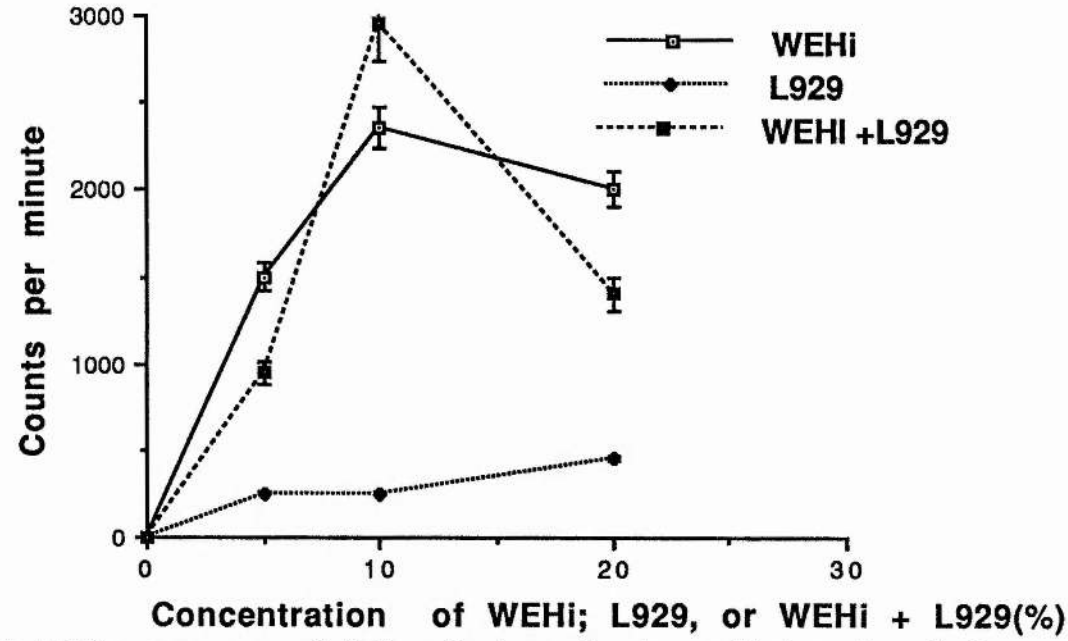


Fig.75:The response of B.M cells from the low cell dose tranplant cell line (SA7FT6) to growth factors monitored in vitro using (3H)-thymidine uptake assay.

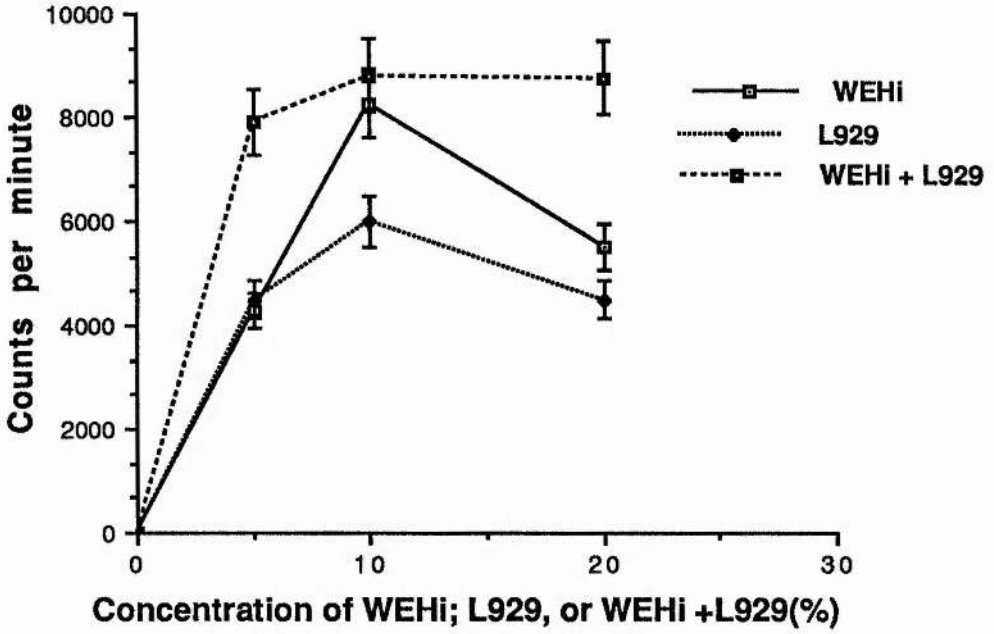


Fig.76: The response of bone marrow cells from the SA8 high cell dose transplant cell line to growth factors monitored in vitro using(3H)-thymidine uptake assay.

Results: Figure 77 shows that leukaemic bone marrow cells from the SA2 cell line were capable of proliferating in medium containing serum in absence of any exogenously added grow factors (0%). When WEHi conditioned medium was added, proliferation (manifested by increased [3H]TdR uptake) increased in a concentration dependent manner and reaches a peak when 10% WEHi was added. Increasing the WEHi concentration to 20% decreased proliferation to below when no growth factor(s) was added. In contrast, addition of L929 conditioned medium resulted in inhibition of proliferation (decreased [3H]TdR uptake) in a concentration dependent manner. Interestingly however, the combination of WEHi and L929 resulted in a synergistic proliferative response. In serum free medium, no proliferation was observed in vitro unless growth factors were added (Figure 78). 10% horse serum was as

effective as 10% foetal calf serum in providing proliferative stimulus in vitro. In the absence of serum, single growth factor responses were not as marked as compared to SA8 leukaemic cell line, probably because serum was supplying other nutrients as well. However, synergistic effects were still manifested with combinations of WEHi and L929 conditioned media. With 10% WEHi and 10% L929; 20% WEHi + 20% L929, in serum free medium, the proliferative responses were greater than that observed in the presence of 10% serum . Transferrin (with or without iron) was no substitute for serum or growth factors in stimulating in vitro proliferation (Figure 79). When transferrin (with or without iron) was added to WEHi (Figure 79) or L929 (Figure 80) in serum free medium, no increased proliferative responses were manifested as compared to when no transferrin was added. Similarly, when the cells were suspended in medium containing transferrin (saturated with iron) with growth factors added, no increased proliferative response was observed (Figure 81). In addition, the synergism observed when the two growth factors were combined in the presence of transferrin (Figure 81) was less pronounced as compared to when no transferrin was present (Figure 78).

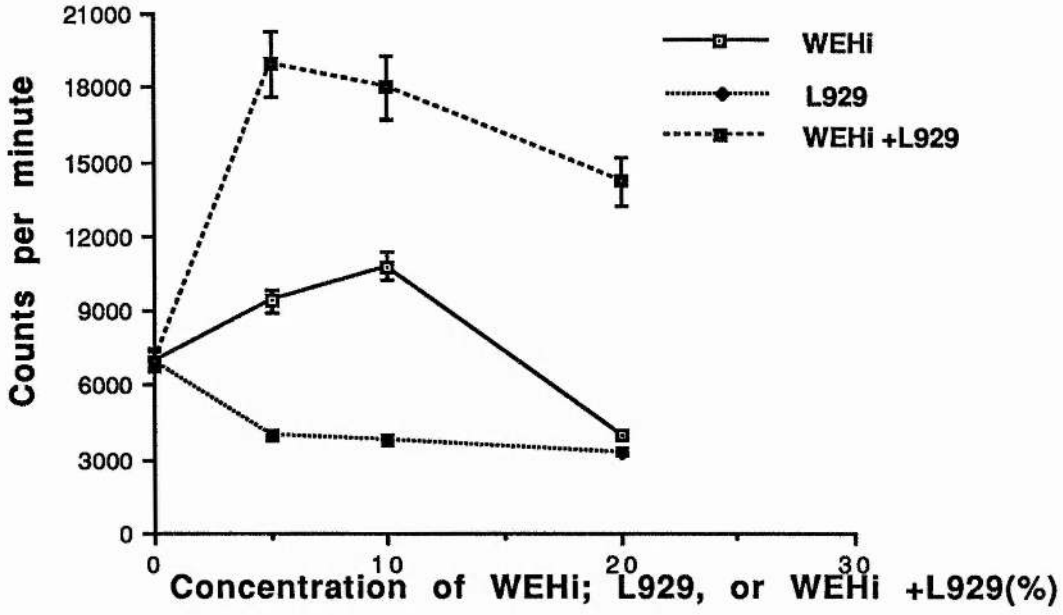


Fig.77:The response of SA2 cell line to growth factors monitored using (3H)-thymidine uptake assay.
(The leukaemic cells were suspended in medium with serum.)

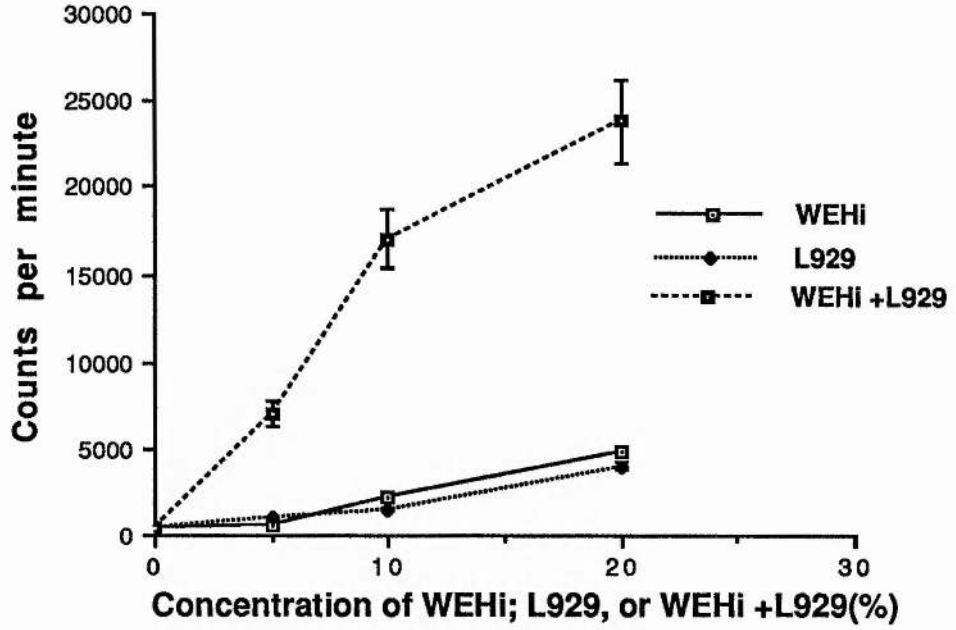


Fig.78: The response of SA2 leukaemic cell line to growth factors in vitro.
(The leukaemic cells were suspended in serum free medium.
1% serum was added to control cells that received no growth factors.)

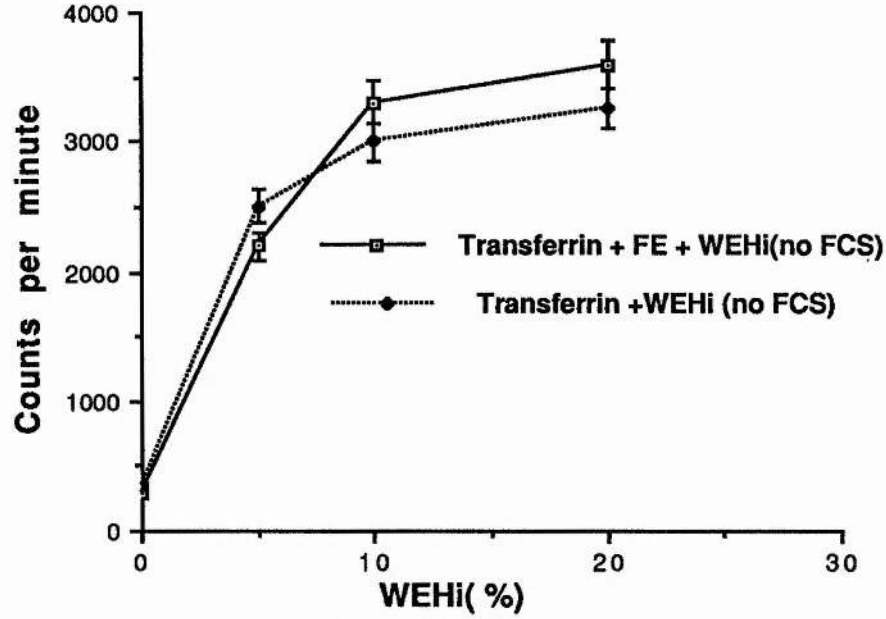


Fig.79: The response of SA2 cell line to WEHi and transferrin (with or without iron) in serum- free medium monitored using(3H)-thymidine uptake assay.

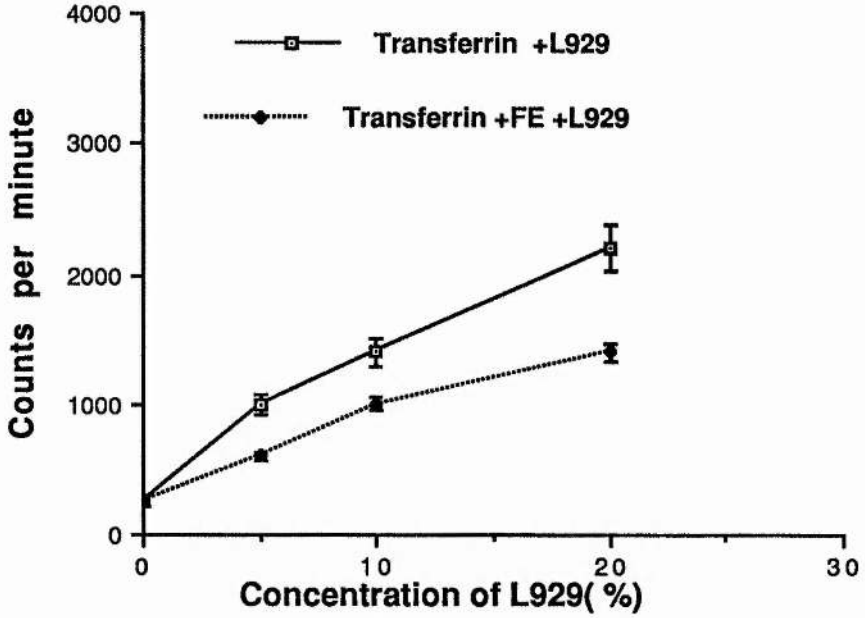


Fig. 80: The response of SA2 cell line to L929 and transferrin (with or without iron) in serum free medium monitored using (3H)-thymidine uptake assay.

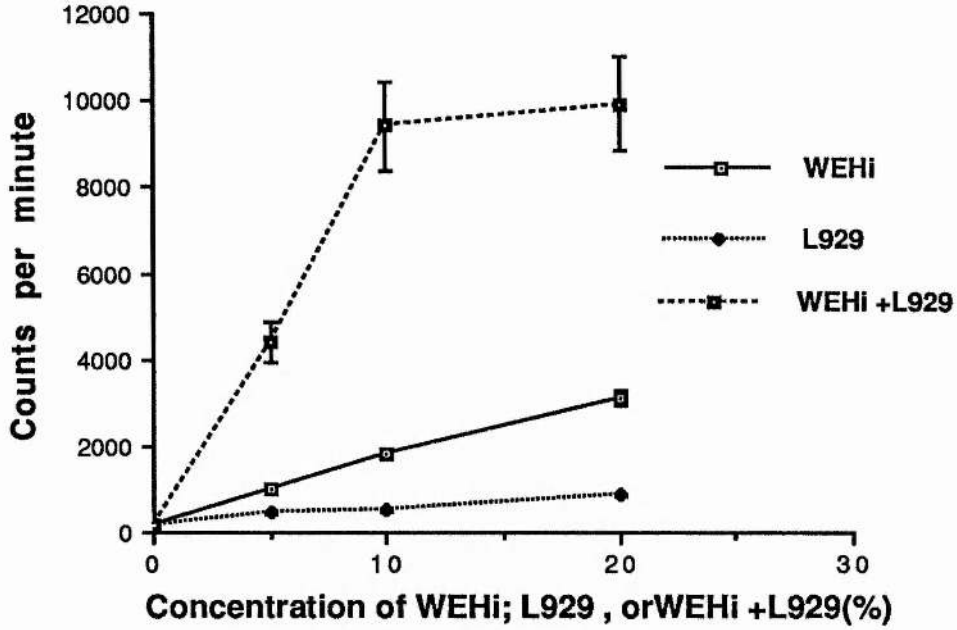


Fig.81: The response of SA2 leukaemic cell line to growth factors in vitro. The cells were suspended in serum -free medium containing transferrin with iron.

5. RESULTS

CHAPTER FIVE

5. In Vivo Chemotherapeutic Studies

In this chapter, the results of the following investigations are reported :

-The responses of low cell dose transplant and high cell dose transplant of SA7 cell line to mitoxantrone administered in vivo were monitored (section 5.1).

-The in vitro growth factor responses of untreated and relapsed leukaemic bone marrow cells of the SA7 high cell dose transplant cell line were studied (section 5.2).

-The responses of bone marrow cells of cured mice to growth factors and drug treatment in vitro were investigated.

-Normal mice received a similar dose and schedule of mitoxantrone as was employed in treating leukaemia bearing mice. The toxicity of the drug on the haematopoietic system was assessed using parameters such as femur cellularity, spleen weight, haemoglobin (Hb) count, haematocrit (packed cell volume, PCV) and in vitro granulocyte-macrophage colony forming cell numbers per 5×10^4 bone marrow cells (section 5.3).

5.1 Responses of Low Cell Dose and High Cell Dose Transplants of SA7 Cell Line to Mitoxantrone Treatment In Vivo: Mice bearing the low cell dose transplant and high cell dose transplant sublines of the SA7 leukaemic cell line were treated with mitoxantrone in vivo. The increase in life span of treated mice was used as a therapeutic end point.

Experimental procedure:

High Cell Dose Transplant: Groups of five female CBA/H mice weighing 20-25g were inoculated with 10^5 leukaemic cells either intravenously (I.V) or intraperitoneally (I.P). On days one and five following tumour inoculation, mice were administered the following doses of mitoxantrone (freshly diluted in normal saline) I.P : 0.75, 1.5, 2 and 3mg/Kg. Control mice were administered leukaemic cells and saline only. The experiment was repeated.

Low Cell Dose Transplant: Similar number of mice (5 / group) and schedule as described above was followed, except that the leukaemic cells were only injected I.V. The following doses of mitoxantrone were injected I.P : 0.4, 0.75 and 1.5mg/Kg.

The survival of treated mice relative to control mice from day of tumour inoculation was determined and the percent increase in life-span was calculated (section 2.6.1). Mice were observed daily and were autopsied when sick in order to determine cause of death. Mice that survived for 60 days (high cell dose transplant) or 90 days (low cell dose transplant) were deemed cured if no evidence of leukaemia was found on autopsy. In addition, bone marrow cytology, cellularity and peripheral blood picture must all be normal. The drug responses (in vitro) of bone marrow cells from cured mice and those dying from drug toxicity were investigated.

Results:

Chemotherapeutic Responses of Low Cell Dose and High Cell Dose Transplants:

Table 1 shows the response of SA7 high cell dose transplant cell line to mitoxantrone when both leukaemic cells and drug were administered intraperitoneally (IP-IP). Within the dose range 0.75-1.5mg/Kg, there

TABLE 1: The response of SA7 high cell dose transplant cell line to mitoxantrone treatment in vivo.

Mice were innoculated with 10^5 leukaemic cells (I.P), and on days 1 and 5 following tumour innoculation they recieved mitoxantrone (I.P) in the doses indicated.(IP-IP)

Dose (mg/kg)	*% ILS* [a]	*Number of 60-day surviviors*
0.75	62.5 (13c)[b]	1/5
1.5	81.5 (146)	1/5
2	60 (128)[c]	1/5
3	1[d]	0/5

a. Mean survival of saline-treated control was 12 days.

b. Increase in life span calculated to include 60-day survivors.

c. Only one of the mice from this group died from leukaemia, the rest died from drug-related toxicity.

d. All animals in this group died from drug toxicity.

was an increase in life-span with increasing dose although not proportionately. This is seen from the observation that doubling the dose from 0.75mg/Kg to 1.5mg/Kg was only accompanied by a 20% increase in life-span or even 10% increase if 60 day survivors were taken into account (Table 1). Similarly, no dose-response relationship was observed in the number of long term survivors following in vivo treatment. Thus, whether 0.75mg/kg or 2mg/kg was administered, only one long-term survivor resulted in each case. Doses above 2mg/kg were toxic. All leukaemia bearing mice that received two doses of 3mg/kg mitoxantrone died from drug-related toxicity as no evidence of leukaemic infiltration (particularly of the spleen) was seen on autopsy (Plate 4). In contrast, there was a decrease in femur cellularity and spleen weight in these mice. Identical results were obtained when the experiment was repeated.

The route of administration of leukaemic cells had a profound bearing on therapeutic outcome. For example, when the leukaemic cells were administered I.V and the drug I.P (IV-IP), there was a sharp decrease in life span of mice as compared to when both leukaemia and drug were administered I.P (Table 2). More than 50% decrease in life-span was observed within the dose range 0.75-1.5mg/Kg with the IV-IP regime as compared to the IP-IP regime. With the latter, most of the mice that received 2mg/Kg mitoxantrone died of drug toxicity as no evidence of leukaemia was seen on autopsy. In contrast, all mice that received mitoxantrone 2mg/kg using the IV-IP regime died as a result of leukaemia. This strongly suggests that survival of the leukaemia bearing mice not only depended on the dose of mitoxantrone administered, but also the mode of leukaemic inoculation.

In the light of the effect of route of administration of leukaemia on therapeutic outcome, any comparison of the therapeutic response of low

Table 2 : The Response of SA7 high cell dose transplant cell line to mitoxantrone treatment in vivo.

Mice were injected with 10^5 leukaemic cells intravenously(I.V). On days 1 and 5 following tumour innoculation, they received mitoxantrone (I.P) in the doses indicated(IV-IP).

Dose mg/kg	*% ILS*[a]	*Number of 60-day surviviors*
0.75	12.2	0/5
1.5	37.5	0/5
2.0	50.0	0/5[b]

a. Mean survival of saline treated controls was 8 days.

b. None of the mice in this group died from drug-related toxicity (ie all died from leukaemia).

cell dose transplant and high cell dose transplant cell lines will have to take this into consideration. The low cell dose transplant cell line (IV-IP) seemed more sensitive to mitoxantrone (Table 3) as compared to the high cell dose transplant (IV-IP) (Table 2). This is despite the observation that the low cell dose transplant grows slower than the high cell dose transplant (compare Tables 2(a) and 3(a)). In addition to the observation that overall increase in life-span produced by identical doses of mitoxantrone were higher in the low cell dose transplant as compared to the high cell dose transplant, there was also one long term survivor observed. No long term survivors were seen from the high cell dose transplant cell line (Table 2) using the IV-IP regime. There was an unexpected incidence of leukaemia relapse which occurred 90 days following therapy in the low cell dose transplant cell line. No such observation was made with the high cell dose transplant cell line.

All other autopsied long term survivors had normal spleen weight (65 ± 3mg) haemoglobin count (15g/L) and haematocrit (45%). This is in contrast to the massively enlarged (leukaemia infiltrated) spleen, reduced haemoglobin and haematocrit values (Tables 4 and 5) seen in terminally leukaemic mice from both the high cell dose passage (Plate 6) and the low cell dose transplant cell lines . During the terminal stage, the low cell dose transplant cell line produced greater effect on haemoglobin (2.8±0.5 vs. 11±0.7) and haematocrit (5±0.9 vs. 31±2.5) values as compared to the high cell dose transplant cell line (Tables 4 and 5).

Responses of Bone Marrow Cells from Cured Mice to In Vitro Treatment with Ara-C, Mitoxantrone or their Combinations: A common feature of bone marrow cells of cured mice is that they become less sensitive to subsequent in vitro treatment with mitoxantrone alone or in

Table 3 : The Response of SA7 low cell dose transplant cell line to mitoxantrone treatment in vivo.

Mice were injected with mitoxantrone 10^5 cells intravenously. They received mitoxantrone intraperitoneally on days 1 and 5 following tumour innoculation(IV-IP).

Dose (mg/kg)	*% ILS*[a]	*Number of 90-day survivors*
0.4	10	0/5
0.75	40	0/5
1.5	128(146)[b]	2/5[c]

a. Mean survival of saline-treated controls was 29 days.

b. Increase in life-span calculated to include 90-day survivors.

c. One of the two 90-day survivors was discovered upon autopsy to have an enlarged spleen.

Table 4 : The effect of SA7 high cell dose transplant cell line on normal haematopoiesis at presentation and at relapse following treatment with mitoxantrone(in vivo).

SA7 High dose Transplant	*Spleen weight (mg)*	*Haemoglobin (g/L)*	*PCV (%)*
Control (untreated)	402.6±20.7[a]	11±0.7[a]	31±2.5[a]
Recurrent leukaemia following treatment with:			
0.75mg/Kg mitoxantrone	434.5±26[d]	11.95±0.7[a]	32±1.9[a]
1.5mg/Kg mitoxantrone	44.2±12[b]	"	"
	405.0±15[d]		
2mg/Kg mitoxantrone	59.3±15[b]	"	"
	271.7[d]		
60-day survivors	60.5[c]	14.0	45.0

a. Mean ± SE of five separate determinations.

b. Mice died from drug toxicity (i.e no evidence of leukaemia was seen on autopsy)

c. Mice survived for 60 days and were deemed cured.

d. Mice died from leukaemia.

Table 5 : The effect of SA7 low cell dose transplant leukaemic cell line on normal haematopoiesis at presentation and at relapse following treatment with mitoxantrone(in vivo).

SA7 low dose transplant cell line	*Spleen weight (mg)*	*Haemoglobin (g/L)*	*PCV (%)*
Control(untreated)	255.4±23.2[a]	2.8±0.5[a]	5.0±0.9[a]
0.4mg/Kg mitox	350.2±33.6	5.2±0.9	16.8±0.8
0.75mg/Kg mitox	280.4±20.3	2.6±0.2	8.4±0.7
1.5mg/Kg mitox	337.4±25.6	3.0±0.8	10.7±2.0
	357.7	5.8[b]	16[b]
90 day survivor	71.6	15.6	45.0

a. Mean ± SE of five separate determinations.

b. Leukaemic relapse occuring 90 days following treatment.

combination with Ara-C. Figures 82 and 83 show the response (mean of two separate experiments) of bone marrow cells from cured mice following treatment of SA7 high cell dose passage with mitoxantrone (0.75mg/Kg). These bone marrow cells were less sensitive than normal (untreated) bone marrow cells to low concentrations of Ara-C (Figure 82). When mitoxantrone (1.2ng/ml) was added to Ara-C, no additive effect was observed unlike what was observed with bone marrow cells from normal (untreated) mice (Figure 82). Similarly, bone marrow cells from cured mice were less sensitive to mitoxantrone (Figure 83) as compared to bone marrow cells from normal mice. Although there was some variability in degree of insensitivity to high concentration of mitoxantrone (120ng/ml) between different mice, bone marrow cells from all cured mice exhibited this decreased sensitivity. For example, the $I.C_{50}$ of mitoxantrone against normal bone marrow cells was 6ng/ml(Figure 83). In contrast, the $I.C_{50}$ of mitoxantrone against bone marrow cells of cured mice was 105ng/ml (Figure 83). No additive effect was seen when Ara-C (1.2ng/ml) was added to mitoxantrone (Figure 83), although this was not as pronounced as for bone marrow cells from normal (untreated) mice (Figure 83). An essentially identical result was obtained on testing bone marrow cells from cured mice following treatment with mitoxantrone (2mg/Kg). The only difference was that in this case there was no difference in sensitivity to Ara-C between bone marrow cells from cured mice and untreated normal mice (Figure 84). However, when mitoxantrone (1.2ng/ml) was added to Ara-C (0.12-120ng/ml), the cells became markedly insensitive to the combination as compared to untreated NBM cells (Figure 85). The usual decreased sensitivity to mitoxantrone was observed even using the highest concentration (120ng/ml)(Figures 86 and 87). When a combination of WEHi (20%) and L929 (20%) was used to stimulate the <u>in vitro</u>

proliferation of bone marrow from cured mice, there was slightly increased sensitivity to Ara-C (Figure 88), while the effect of mitoxantrone sensitivity depended on the concentration(Figure 89).

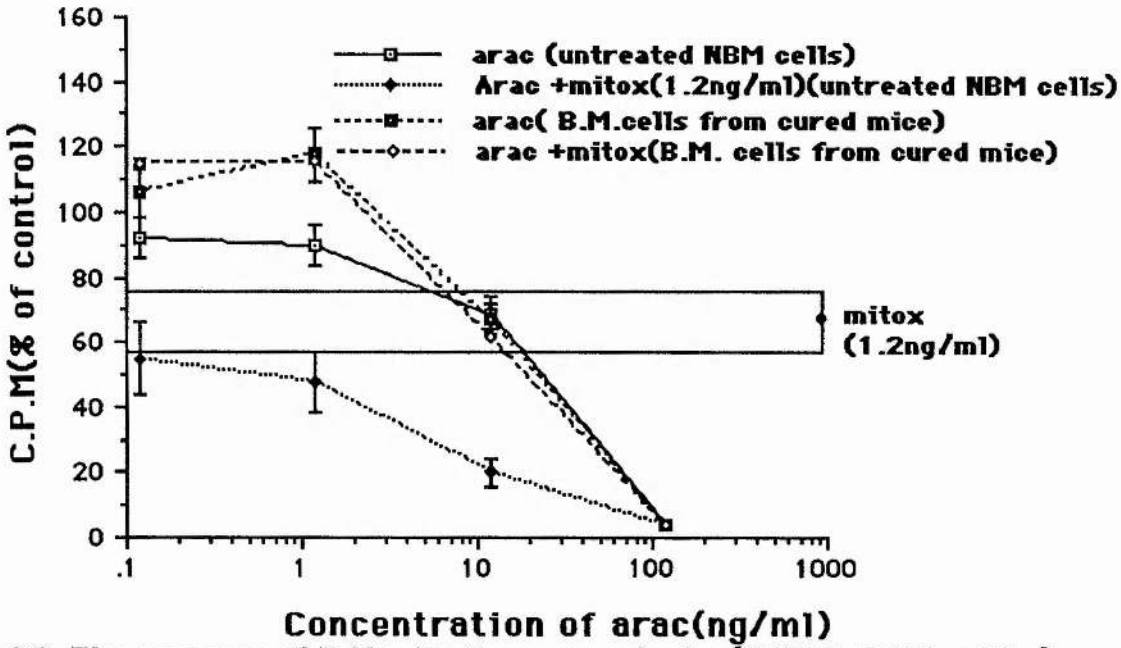


Fig.82: The response of B.M.cells from cured mice(SA7HD;0.75mg/Kg.) to arac treatment in vitro monitored using the (3H)-thymidine uptake assay.

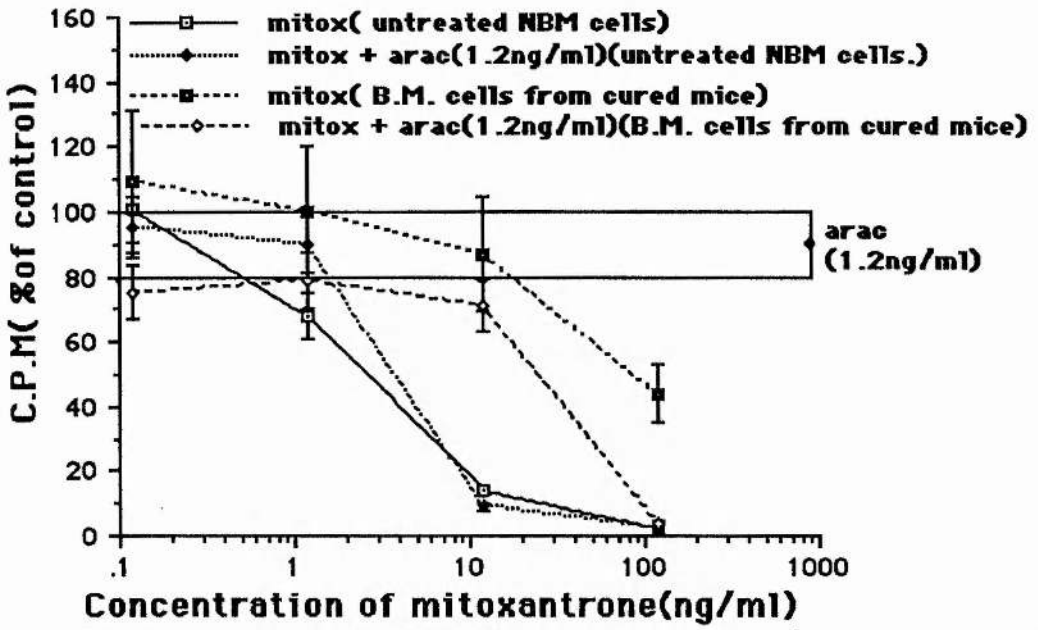


Fig. 83:The response of B.M. cells from cured mice(SA7HD;0.75mg/Kg) to mitoxantrone treatment in vitro. Mitoxantrone I.C. 50 against untreated B.M cells was 6ng/ml, and 105ng/ml for B.M. cells of cured mice.

At low to intermediate concentrations of mitoxantrone (0.12-12ng/ml), there was a slight decrease in sensitivity, while at the highest concentration of mitoxantrone used (120ng/ml) there was slight increase in sensitivity to mitoxantrone (Figure 89). The decreased sensitivity developed by bone marrow cells of cured mice apparently did not depend on the mitoxantrone dose used in the treatment since it was observed following treatments with both 0.75mg/kg and 2mg/kg mitoxantrone. It also did not depend on the type of leukaemia cell line treated, since it

was also observed in bone marrow cells of cured mice following treatment of low cell dose transplant with mitoxantrone (1.5mg/kg). Figure 90 shows the response of these bone marrow cells to Ara-C alone (0.12-120ng/ml) or in combination with mitoxantrone (1.2ng/ml), while Figure 91 shows the response to mitoxantrone (0.12-120ng/ml) in combination with Ara-C (1.2ng/ml) and Figure 92 shows response of the cells to mitoxantrone alone. Responses similar to those described for bone marrow cells of mice cured following treatment of the high cell dose transplant with mitoxantrone were observed. Even myeloid progenitor cells of bone marrow cells from cured mice apparently exhibit decreased sensitivity against subsequent mitoxantrone exposure in vitro (Figure 93). Thus there was an agreement between the colony assay and [3H]TdR uptake assay results.

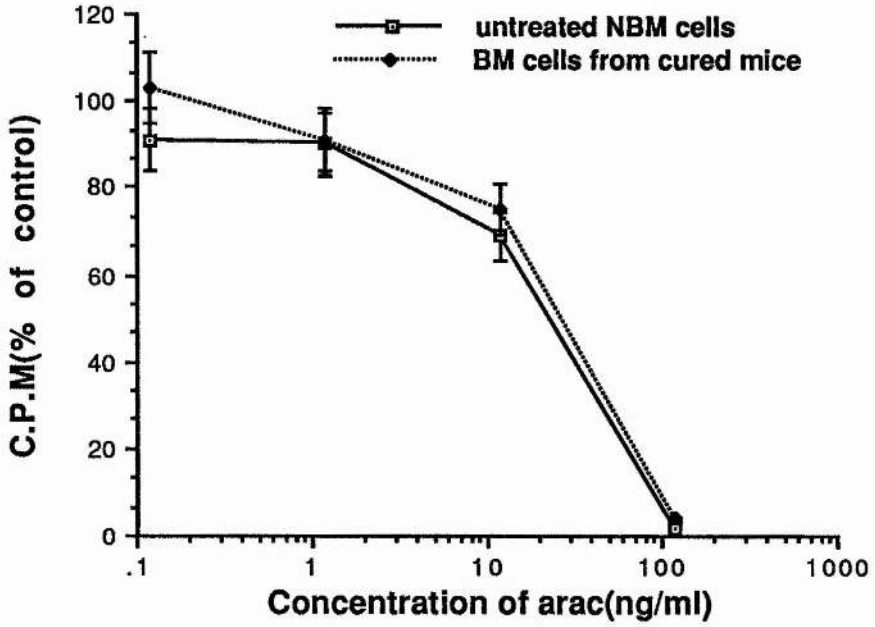


Fig.84: The response of BM cells from cured mice(SA7HD; 2mg/Kg) to arac treatment in vitro monitored using (3H)-thymidine uptake assay.

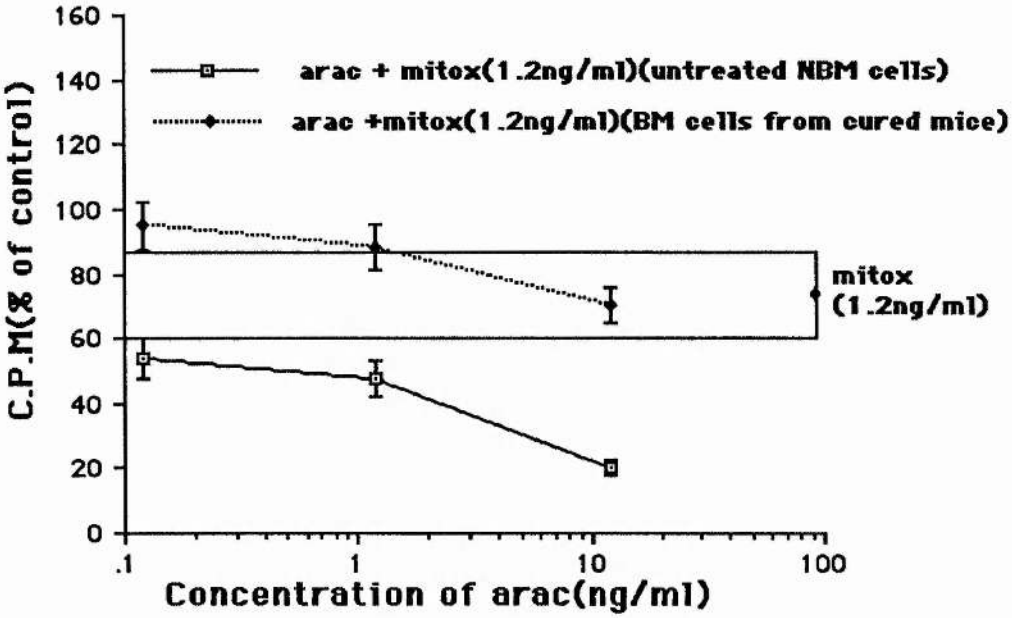


Fig.85: The response of BM cells from cured mice(SA7HD; 2mg/Kg) to combination of arac with mitoxantrone monitored using the (3H)-thymidine uptake assay.

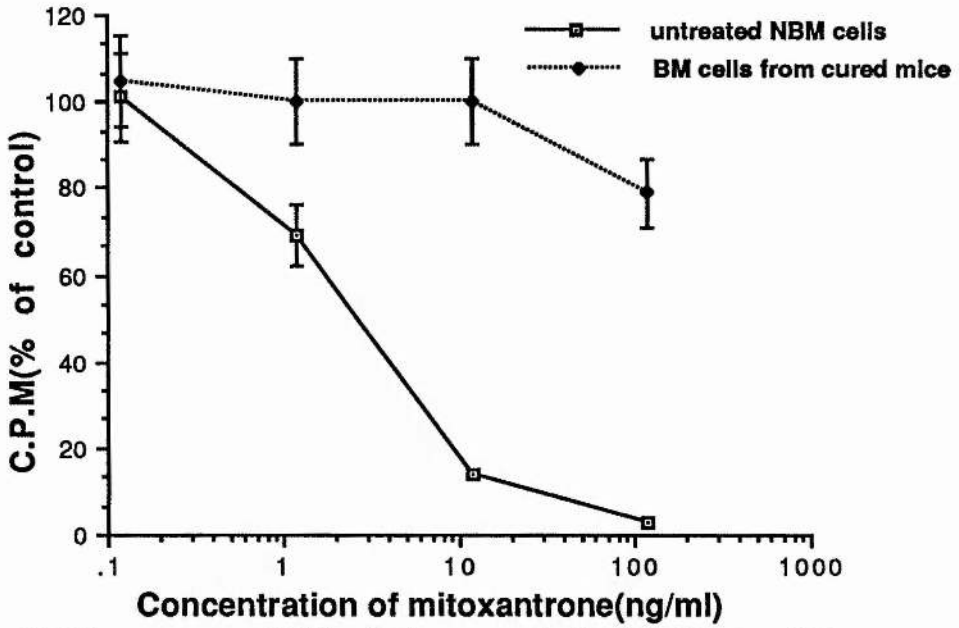


Fig.86: The response of BM cells from cured mice(SA7HD;2mg/Kg) to mitoxantrone treatment in vitro monitored using (3H)-thymidine uptake assay.

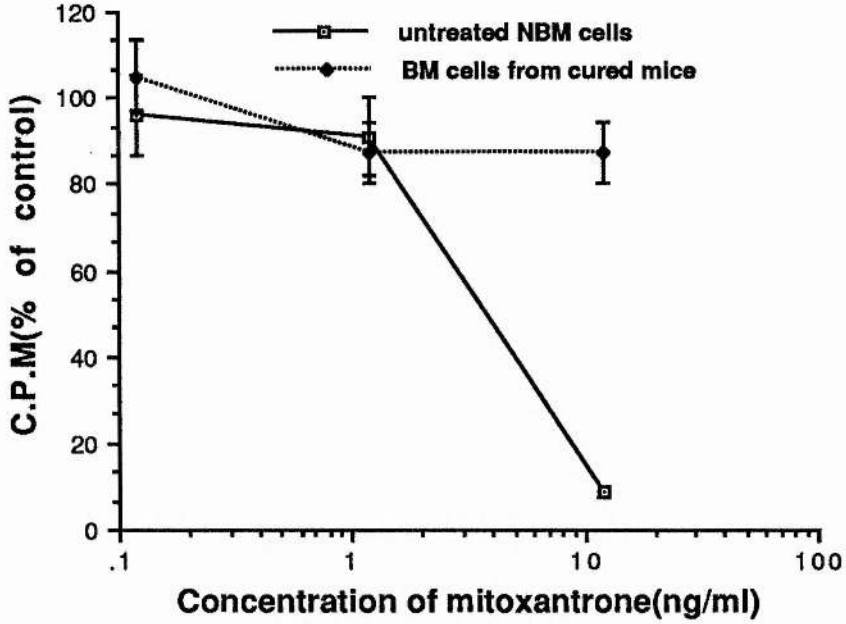


Fig.87: The response of BM cells from cured mice(SA7HD; 2mg/Kg) to mitox treatment in vitro monitored using (3H)-thymidine uptake assay.

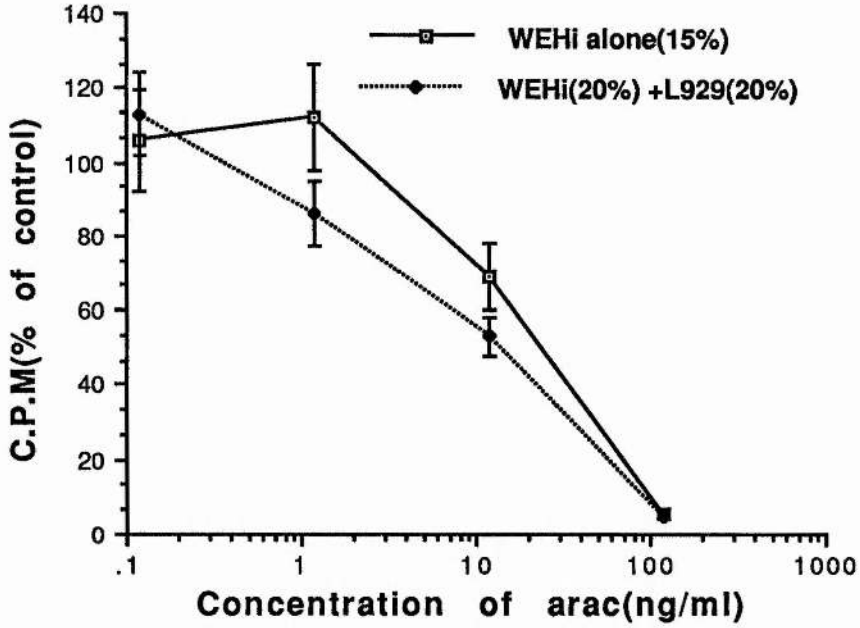


Fig.88: The response to arac treatment in vitro of BM cells from cured mice(SA7HD; 0.75mg/Kg).The cells were stimulated with either WEHi alone or WEHi +L929 in vitro.

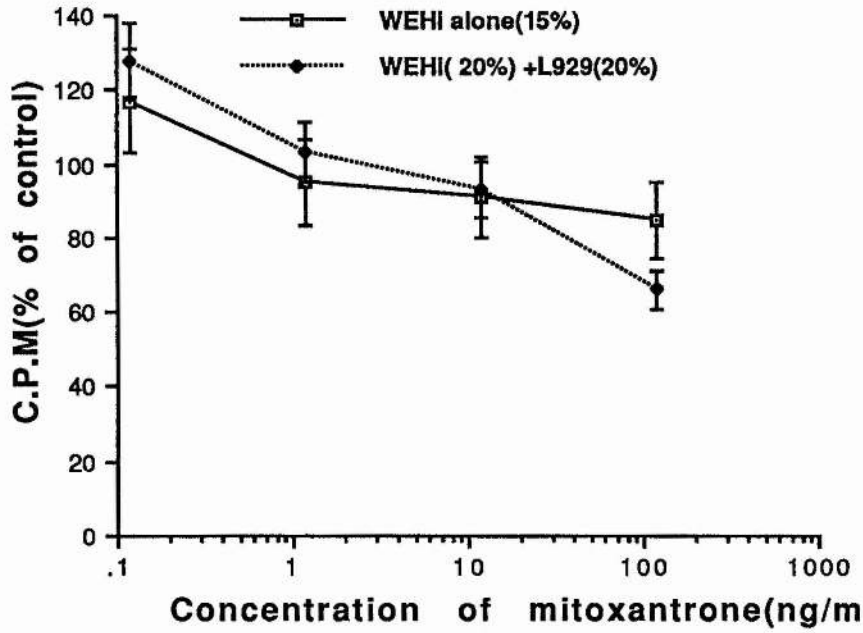


Fig.89: The response to mitox of BM cells from cured mice(SA7HD;0.75mg/Kg) The cells were stimulated with either WEHi alone or WEHi+L929 in vitro.

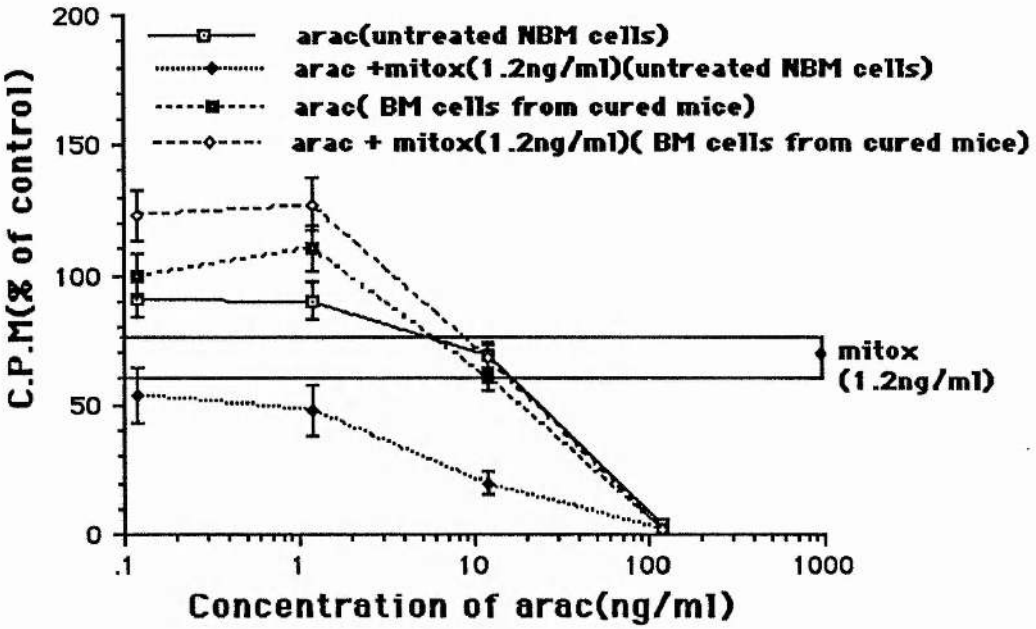


Fig.90: The response of BM cells from cured mice(SA7LD;1.5mg/kg) to arac alone or in combination with mitoxantrone monitored using the (3H)-thymidine uptake assay.

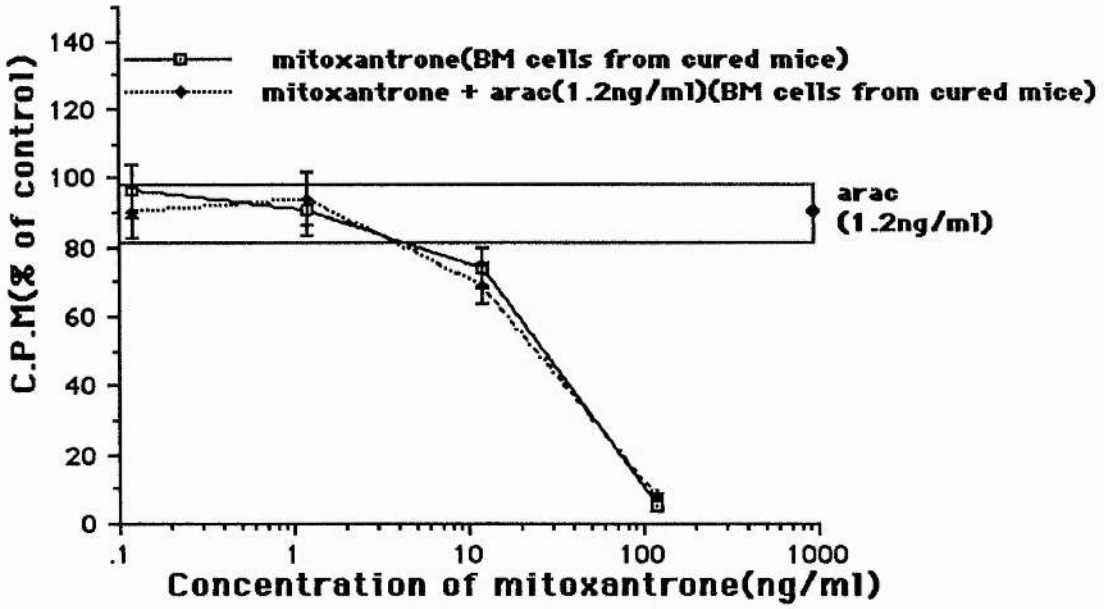


Fig.91: The response of BM cells from cured mice(SA7LD;1.5mg/Kg) to mitoxantrone alone or in combination with arac monitored using the (3H)-thymidine uptake assay.

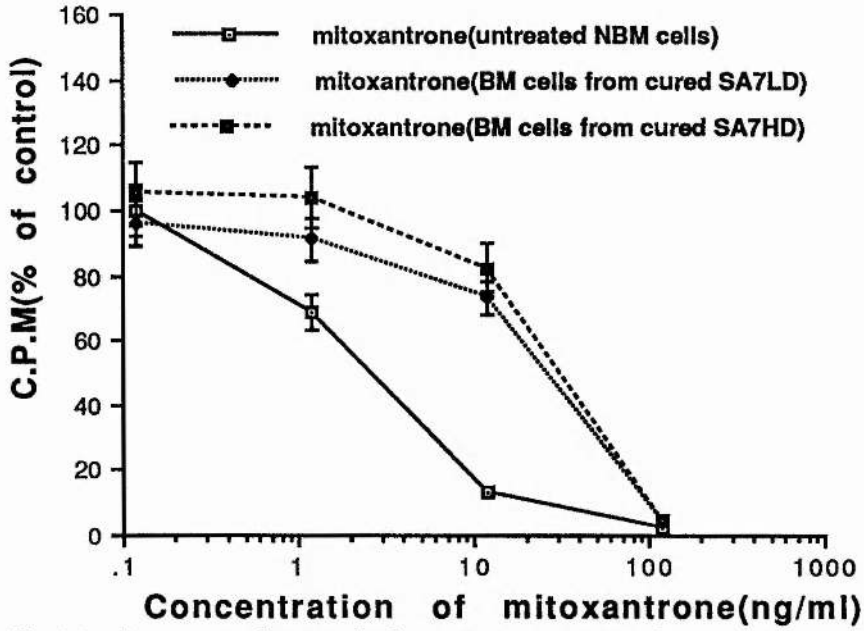


Fig.92: A comparison of the response to mitoxantrone of BM cells from cured mice formally bearing high cell dose and low cell dose passages of SA7 leukaemic cell line monitored using(3H)-thymidine uptake assay.

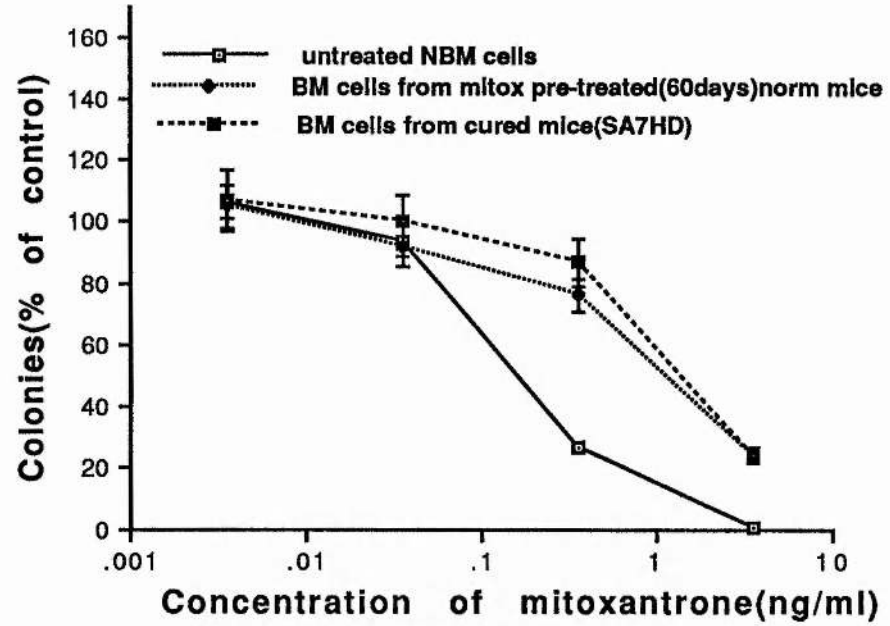


Fig.93: The response to in vitro treatment with mitoxantrone of BM cells from cured(SA7HD) and mitoxantrone treated normal mice. Cytotoxicity was determined using GM-CFC assay.

Response of Bone Marrow Cells of a Mouse Dying from Drug Toxicity due to Drug Treatment In Vivo: Some mice died 30 days following in

vivo treatment of SA7 high cell dose transplant leukaemia with mitoxantrone. On autopsy, no evidence of leukaemic infiltration was seen in bone marrow (Plate 5), spleen and blood. Compare Plate 5 with Plates 6 and 7. The in vitro drug sensitivity of bone marrow cells from one of these mice was tested. No difference in sensitivity to in vitro mitoxantrone treatment was found between such bone marrow cells and bone marrow cells from normal (untreated) mice (Figure 94)(plate 5).

Differential Cell Count of Bone Marrow Cells from Cured Mice: A differential count of bone marrow cells from long term survivors of both low cell dose passage (Tables 6a and 6b), high cell dose passage (Tables 7a and 7b) (Plate 8) and untreated normal mice (Tables 8a and 8b)(Plate 9) shows that the cytology is identical for all three. For example, in all three, the generalised blast count was less than 5%. A blast count of less than 5% is one of the criteria for both successful remission induction and cure in human acute myeloid leukaemia. In addition, the percentages of mature cells (band and segmented cells) were identical in all three. Furthermore, there was no difference in in vitro progenitor numbers per 5×10^4 bone marrow cells between cured and normal mice.

Plate 5: Bone marrow cells from a mouse dying of drug toxicity while in apparent remission . Mice bearing SA7HD leukaemia were treated with mitoxantrone (2mg/Kg).This mouse was autopsied 30 days after drug treatment. No evidence of leukaemic infiltration of the bone marrow is apparent. Spleen was also normal. MM= metamyelocytes; EBL= erythroblast . GB= Generalised blast (Magnification x 1300).

Plate 6: Spleen cells of a mouse bearing SA7HD leukaemia in the terminal stage. The spleen was heavily infiltrated with leukaemic(generalised) blast cells(GB)(Magnification X 1300).

Plate 7: Bone marrow cells of a mouse bearing SA7HD leukaemia in the terminal stage. Most of the cells populating the bone marrow are generalised blasts(GB) with a few mature neutrophils(N)(Magnification x 1300).

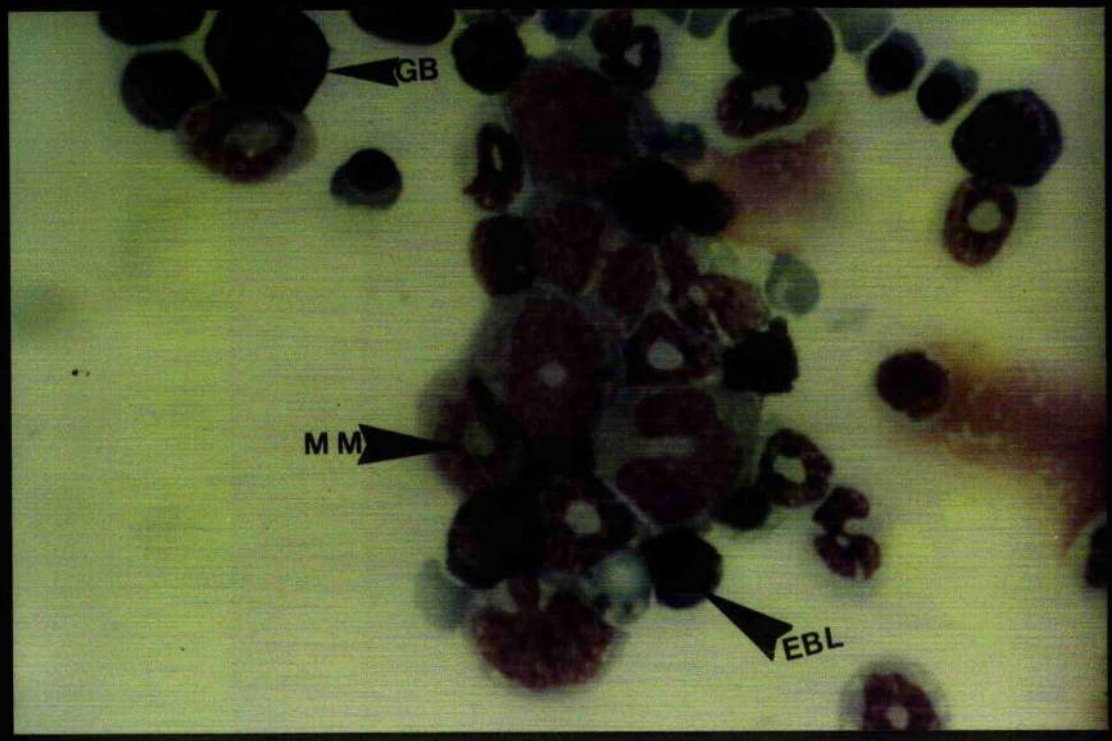


Plate 8: Bone marrow cells from a cured mouse following treatment of SA7HD leukaemia with 2 doses of mitoxantrone(0.75mg/Kg). A normal differential count was obtained. EBL= erythroblast; GB= generalised blast; MM= metamyelocyte (Magnification x 1300).

Table 6a : Differential count of bone marrow cells of a long-term survivor following in vivo treatment of SA7 low cell dose passage(SA7LD) leukaemia with 2 doses of mitoxantrone (1.5mg/kg).

Cell Type	*% Total*	*% Haematopoietic cells*
Generalised blasts	3.41	4.76
Myeloid cells	29.55[a]	41.27[a]
Monocytes	2.27	3.17
Erythroid	32.39	45.24
Lymphocytes	1.42	1.98
Damaged cells	0.57	0.79
Unclassified	0.57	0.79
Other cells	1.42	1.98
Tissue cells	28.41	

a. See Table 6b for further classification of the myeloid cells.

Table 6b : Further classification of the myeloid cells from longterm survivor formerly bearing SA7 low cell dose transplant leukaemia that was treated with mitoxantrone 1.5mg/kg(see table 6a)

Cell type	*%Myeloid*
Myeloblasts	10.34
Promyelocytes	7.76
Myelocytes	9.48
Metamyclocytes	31.90
Band cells	31.90
Segmented cells	8.62

Table 7a : Differential count of bone marrow cells of a cured mouse following in vivo treatment of SA7 high cell dose transplant leukaemia(SA7HD) with 2 doses of mitoxantrone (0.75mg/kg).

Cell type	% *Total*	% *Haematopoietic cells*
Generalized blasts	1.98	2.08
Myeloid cells	39.68[a]	41.67[a]
Monocytes	1.98	2.08
Erythroid cells	31.35	32.92
Lymphocytes	10.71	11.25
Damaged cells	5.16	5.42
Unclassified cells	0.79	0.83
Other cells	3.57	3.75
Tissue cells	4.76	-

a. See Table 7b for further classification of myeloid cells.

Table 7b:Further classification of myeloid cells from bone marrow cells described in Table 7a.

Cell Type	*% Myeloid*
Myeloblasts	5.15
Promyelocytes	5.15
Myelocytes	9.28
Metamyelocytes	42.27
Band cells	35.05
Segmented cells	3.09

Table 8 a : A differential count of normal bone marrow cells from CBA/H mice

Cell type	*% Total*	*% Haem*
Generalised blasts	1.5	1.7
Myeloid cells	5.4	41.0[a]
Monocytes	0.8	1.9
Erythroid blasts	1.8	22.0
Lymphocytes	1.8	16.0
Damaged cells	2.5	2.2
Unclassified cells	0.9	0.4
Other cells	0.8	1.2
Stromal cells	3.8	1.3

a. See Table 8b for further classification of myeloid cells.

Table 8b : Further classification of myeloid cells from bone marrow cells described in Table 8a.

Cell type	*% Total*	*% Haem*
Generalised blasts	3	5.2
Promyelocytes	2.6	5.2
Myelocytes	11	9.4
Metamyelocyte	46	44
Band cells	32	30
Segmented cells	6	6.5

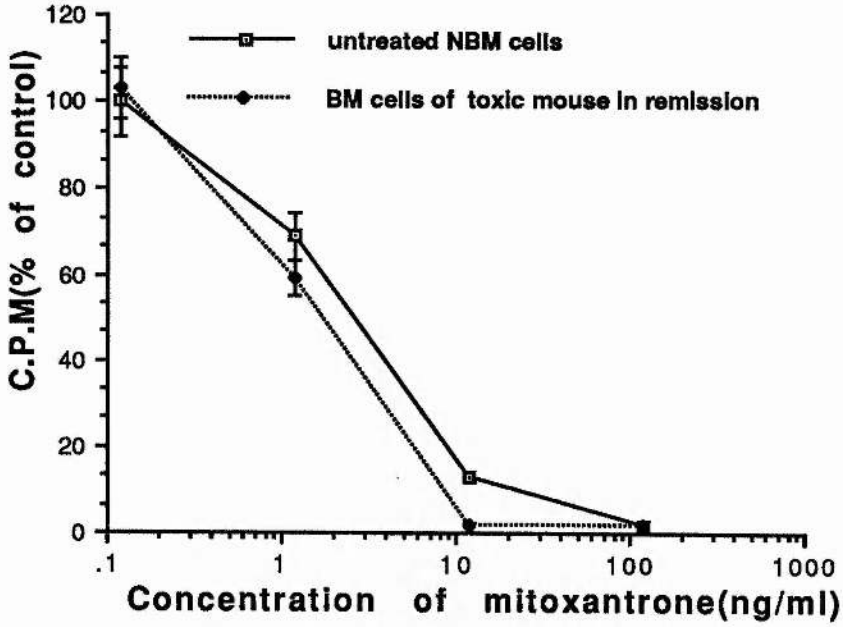


Fig.94: The response of BM cells from untreated normal mice and mouse dying from drug toxicity in remission to mitoxantrone treatment in vitro. Cytotoxicity was monitored using (3H)-thymidine uptake assay.

5.2 Responses of Recurrent Leukaemic Cells to Growth Factors: The growth factor sensitivity of SA7 high cell dose transplant leukaemic bone marrow cells before treatment, and at relapse following in vivo treatment with mitoxantrone, were monitored (Section 5.2.2). Relapsed (recurrent) leukaemic cells were then passaged in (a) normal (untreated) mice and (b) mitoxantrone pretreated normal mice. The in vitro growth factor sensitivity of the leukaemic cells after every passage was determined.

5.2.1 Experimental Procedure: Models of recurrent (rec) leukaemic (leuk) disease following chemotherapy with mitoxantrone were obtained as follows: Groups of 5-6 normal mice of similar age, weight and sex as described under Section 5.1 were injected (I.P) with 10^6 leukaemic cells of the SA7 high cell dose passage cell line and mitoxantrone was administered (I.P) on days 1 and 5 following tumour inoculation in the

following doses: 0.4, 0.7 and 1.5mg/Kg. Alternatively, the leukaemic cells (10^6) were injected I.V and the drug in the following doses was injected I.P: 0.7, 1.5 and 2mg/kg. The leukaemias that recur despite treatment (called recurrent leukaemias) were tested for growth factor (WEHi, L929, WEHi+L929) sensitivity in vitro using procedures already described (section 4.1). In addition, the number of in vitro colony forming cells in recurrent leukaemic bone marrow cells were also determined (section 5.2.8). This was done using the granulocyte-macrophage colony forming cell assay (section 3.4). Briefly, a single cell suspension of recurrent leukaemic bone marrow cells was prepared and 5×10^4 cells were plated in agar and the colonies that developed after 7 days incubation were scored using an inverted microscope. Additionally, the following were carried out:

-Mice bearing the SA7 high cell dose leukaemia were treated by a single dose of mitoxantrone (0.75mg/Kg) either 24 hours or 48 hours before they became moribund with leukaemia. The growth factor sensitivity of the leukaemic bone marrow cells was then determined at autopsy (Section 5.2.3).

- Normal mice were administered mitoxantrone (0.75mg/kg) and autopsied 48 hours later. Similarly, normal mice were administered the following doses of mitoxantrone: 0.4, 0.75, 2 and 3mg/kg. The mice were autopsied and the growth factor sensitivity of bone marrow cells determined (section 5.2.4.).

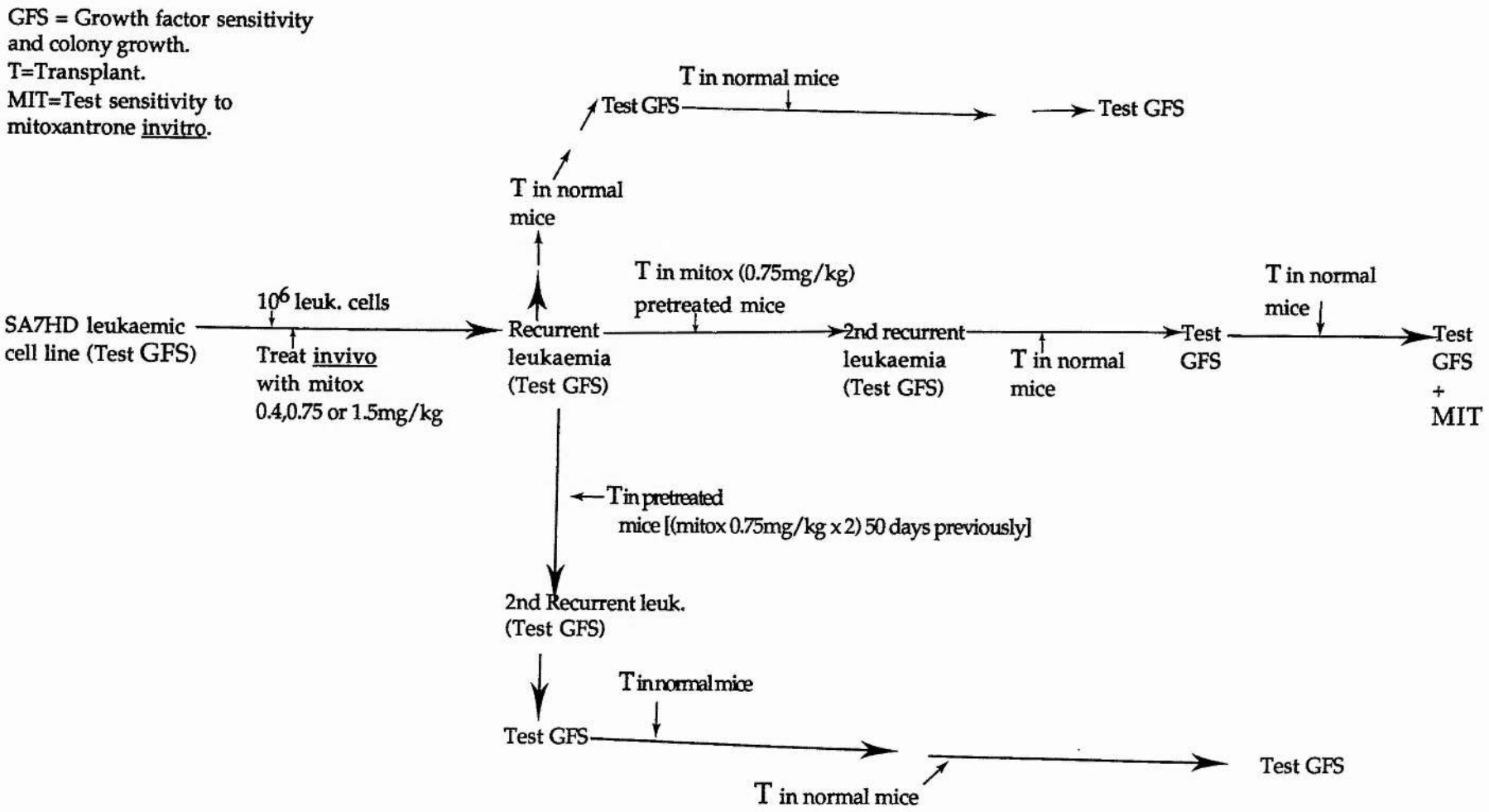
- The bone marrow cells of leukaemic mice dying (while in remission) of drug toxicity (30 days after drug administration) were tested for growth factor sensitivity in vitro (section 5.2.5.).

- Bone marrow cells of cured mice and mice with relapsed low cell dose leukaemia (SA7LD)(which occurred after 90 days) were tested for their growth factor sensitivity in vitro (section 5.2.6). In addition, normal

mice received 2 doses of mitoxantrone (0.75mg/kg) on days 1 and 5. They were autopsied 60 days later and the sensitivity of the bone marrow cells to growth factors was monitored (section 5.2.6.).

- Recurrent SA7 high cell dose (SA7HD) leukaemic bone marrow cells were co-cultured with normal bone marrow cells. The growth factor sensitivity of the mixed population was determined (section 5.2.7).
- Recurrent leukaemic bone marrow cells were plated in agar and colony growth was evaluated after seven days incubation (Section 5.2.8).
- Recurrent (SA7HD) leukaemic cells were passaged into (a) normal mice (b) mice that received single dose of mitoxantrone (0.75mg/kg) 2 days previously (given the code PASS MIT 2d) or 2 doses of mitoxantrone (0.75mg/kg) 50 days previously (given the code PASS MIT 50d) (see Figure 95). The leukaemias from (a) and (b) (called 2nd recurrent leukaemias) were tested for growth factor sensitivity in vitro and then further passaged two times (in normal untreated mice), testing for in vitro growth factor response and myeloid progenitor numbers after each passage (Figure 95). After the second passage, the response of the leukaemic cells to mitoxantrone treatment in vitro was also investigated (Figure 95) (sections 5.2.9 - 5.2.20).
- The spleens of mitoxantrone pretreated mice with (2nd) recurrent leukaemia were weighed and compared to those of untreated mice with (2nd) recurrent leukaemia (5.2.21).
- The in vitro mitoxantrone sensitivity of recurrent leukaemic cells after passage in normal mice was determined (section 5.2.22). Similarly, the sensitivity of the (2nd) recurrent leukaemic cells following passage of recurrent leukaemia in mitoxantrone pretreated mice was monitored (section 5.2.23).
- A differential count of recurrent leukaemic and untreated leukaemic bone marrow cells was performed (section 5.2.24).

Figure 95 : Flow diagram of growth factor sensitivity tests on recurrent leukaemias.



Results:

5.2.2 Growth Factor Sensitivity of Recurrent Leukaemic Cells: When mice bearing SA7 high cell dose(SA7HD) leukaemia were treated with mitoxantrone, there was a dose-dependent increase in life-span of the treated mice within the dose range 0.75-1.5mg/Kg. Although some mice were even cured by these doses, in many mice the disease recurred. Leukaemic bone marrow cells from mice with recurrent leukaemia were not responsive to growth factors in vitro. Figure 96 shows the response of both untreated SA7HD leukaemic bone marrow cells and recurrent (SA7HD) leukaemic bone marrow cells to WEHi (mean of 3-6 separate experiments with 2-3 mice per experiment). While untreated leukaemic cells were responsive to WEHi, recurrent leukaemic cells (as a result of treatment with mitoxantrone 0.4, 0.75, and 1.5mg/Kg) did not respond. Similarly, Figures 97 and 98 show (respectively) the responses of untreated and recurrent leukaemic cells to L929 alone and combinations of WEHi and L929. Again while untreated leukaemic cells were responsive to L929 alone and showed synergistic proliferative response to combinations of WEHi and L929, the recurrent leukaemic cells were insensitive to L929 alone or even combinations of L929 and WEHi conditioned media. The growth factor responses of recurrent leukaemic cells were significantly ($P=0.04$) different from those of untreated leukaemic cells using the Mann-Whitney test. As was observed with drug treatment, there was a difference in growth factor sensitivity when the leukaemic cells were injected intravenously with mitoxantrone administered intraperitoneally. In this case the recurrent leukaemic cells retained minimal response to WEHi (Figure 99), L929 (Figure 100) and combinations of WEHi and L929 (Figure 101).

5.2.3 Growth Factor Responses of Bone Marrow Cells from Leukaemic Mice Treated with Mitoxantrone 24 or 48 hours before they became Moribund with Leukaemia: When mitoxantrone(0.75mg/Kg) was administered to leukaemic mice 24 or 48 hours before they became moribund with the disease, the leukaemic cells retained sensitivity to growth factors in vitro.

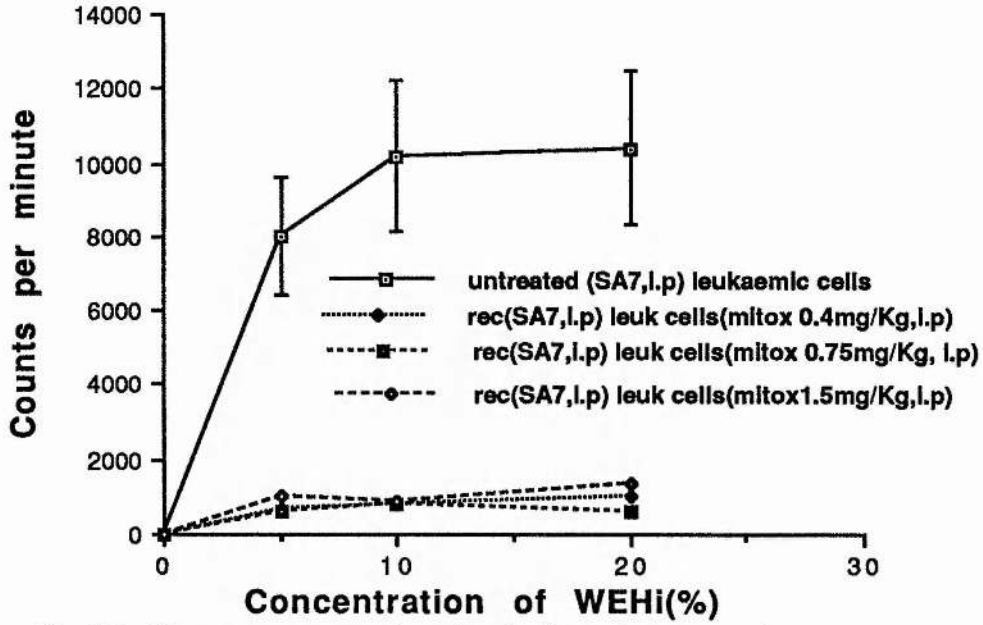


Fig.96: The response of untreated and recurrent SA7HD leukaemic cell line to WEHi. Growth factor response was monitored using (3H)-thymidine uptake assay.

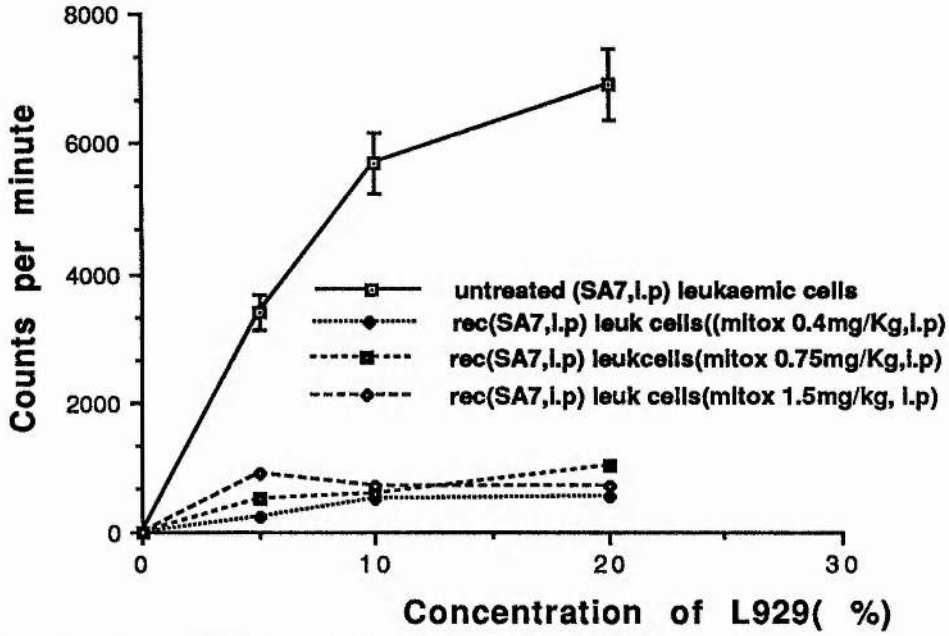


Fig.97: The response of untreated and recurrent(SA7HD) leuk cells to L929. Growth factor response was monitored using(3H)-thymidine uptake assay.

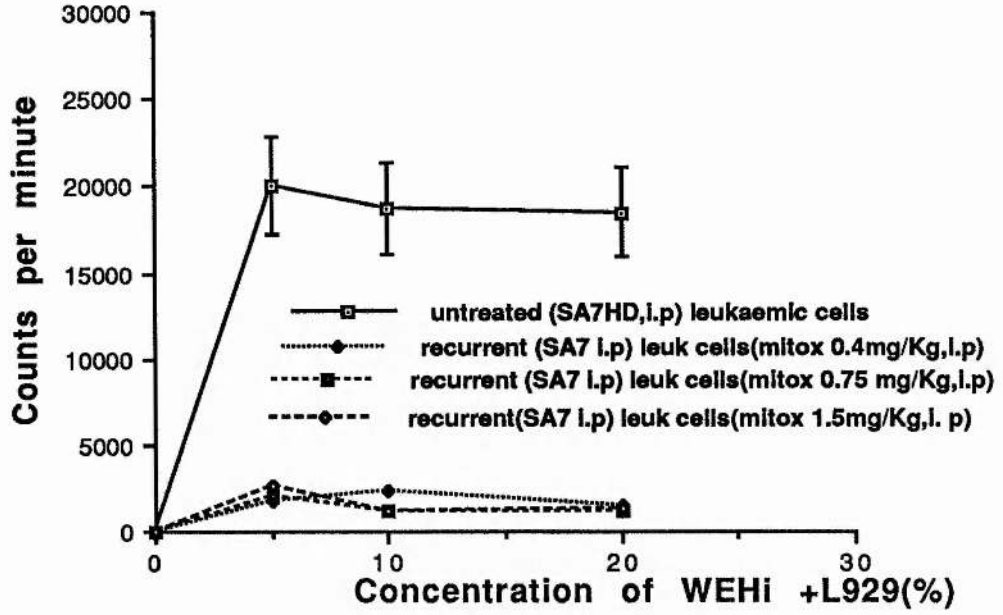


Fig.98: The response of untreated and recurrent(SA7HD) leukaemic cells to WEHi +L929 monitored in vitro using (3H)-thymidine uptake assay.

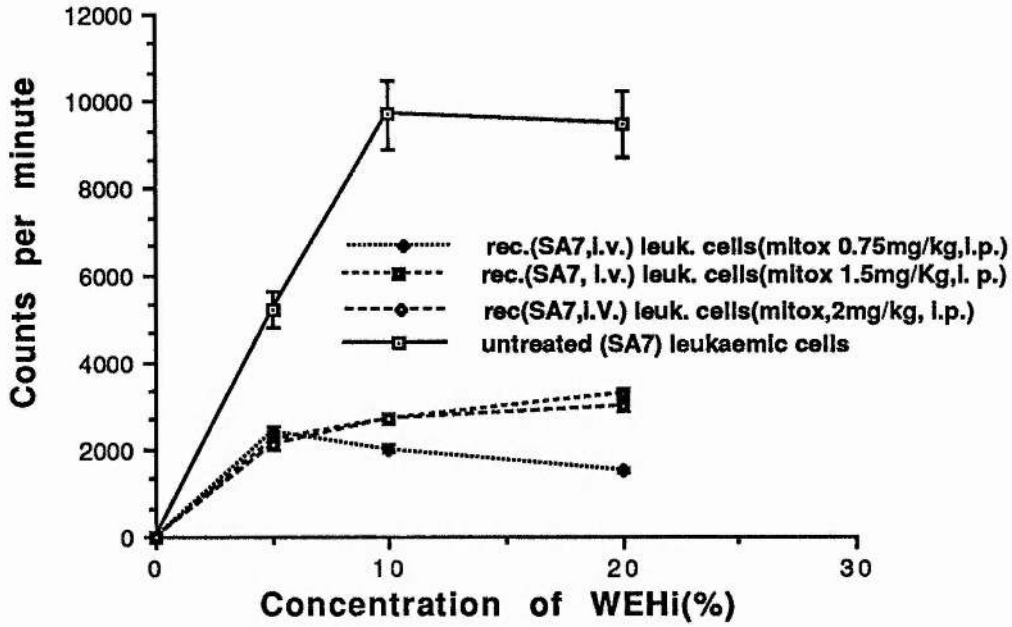


Fig.99: The response of untreated and recurrent leukaemic cells to WEHi. SA7HD Leuk. cells were injected i.V. while mitox. was admin i.p. Growth factor response was monitored using(3H)-thymidine uptake assay.

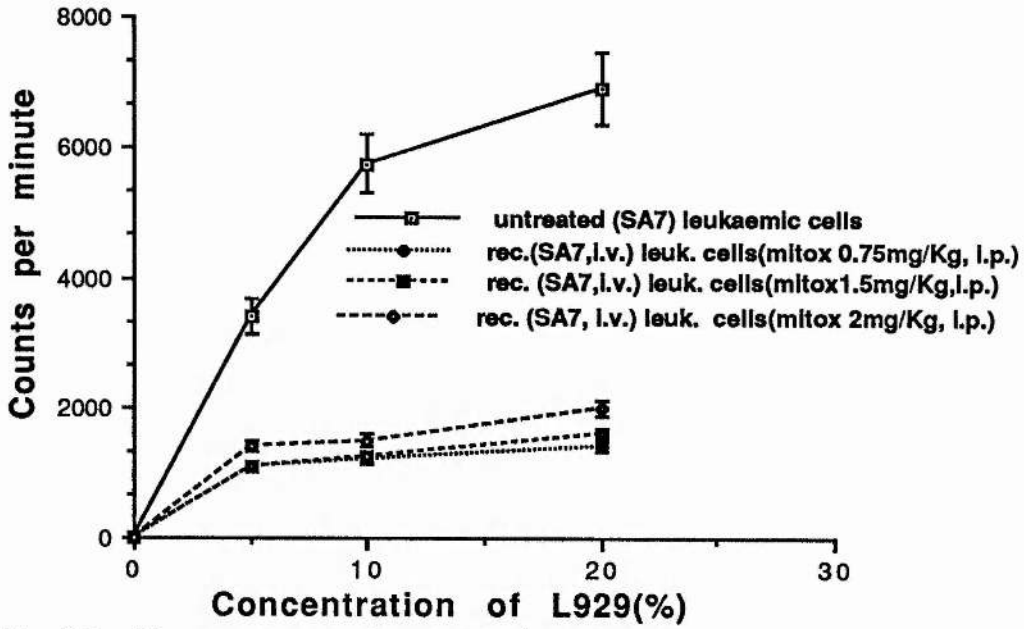


Fig.100: The response of untreated and recurrent leuk cells to L929. SA7HD Leuk cells were injected i.v while mitox was admin i.p. Growth factor response was monitored using (3H)-TdR uptake assay.

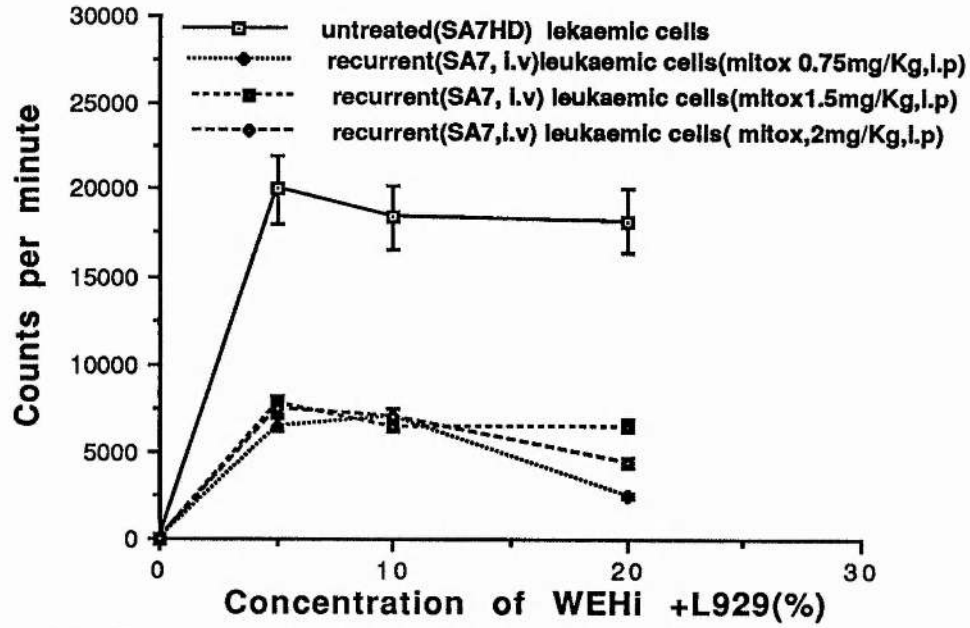


Fig.101: The resp of untreated and rec.(SA7HD) leuk cells to growth factors. SA7HD Leuk. cells were Inj(I.v.) and mitox was admin (I.p.). Growth factor response was monitored following 4days incub using (3H)TdR uptake assay.

The responses of leukaemic cells from mice that received mitoxantrone 24 hours (Figure 102) or 48 hours (Figure 103) are shown. In both cases, the cells were responsive to WEHi alone, L929 alone and synergistic proliferative response was observed with combination of WEHi and L929.

5.2.4 Growth Factor Responses of Bone Marrow Cells from Treated and Untreated Normal Mice: Bone marrow cells of both mitoxantrone (48 hours) treated (Figure 104) and untreated (Figure 105) normal mice responded to growth factors in vitro in a concentration dependent manner with combinations of the growth factors producing synergistic effects. Similarly, bone marrow cells of normal mice treated with mitoxantrone in the doses 0.4mg/Kg (Figure 106); 2mg/Kg (Figure 107) or even 3mg/kg (Figure 108) responded to WEHi, L929 and combinations of WEHi+L929 in the expected manner. Each figure is the mean of three experiments. These doses range from the lowest to the highest that were used in the treatment of SA7 high cell dose transplant leukaemic cells in vivo. Thus, normal bone marrow cells retain their sensitivities to growth factors following treatment of normal mice with mitoxantrone.

5.2.5 Growth Factor Responses of Bone Marrow Cells of a Mouse Dying from Drug Toxicity: In one of the drug treated leukaemia bearing mice, no evidence of leukaemia was found on autopsy 30 days after the initiation of drug treatment, although the mouse was sickly . Death was therefore ascribed to drug-related toxicity. The growth factor response of bone marrow cells of the mouse was monitored. The bone marrow cells were responsive to growth factors in the usual manner (Figure 109) except that sensitivity to 5% L929 and 10% L929 was increased probably as a result of compensatory mechanisms.

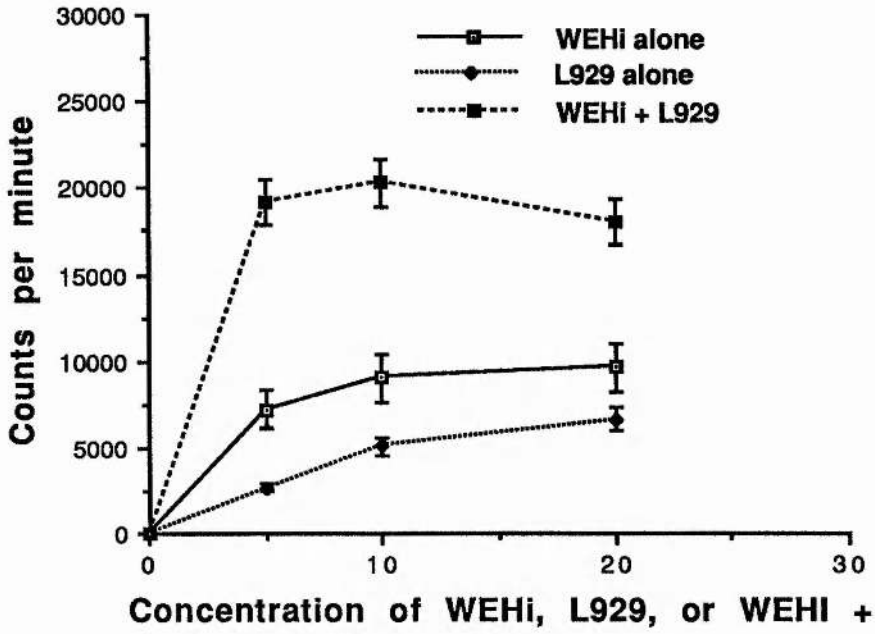


Fig.102:The Response of in vivo mitox treated leuk cells to growth factors. SA7HD Leuk. mice received mitox(0.75mg/Kg) 24hrs before they were moribound Growth factor response was monitored using (3H)-thymidine uptake assay.

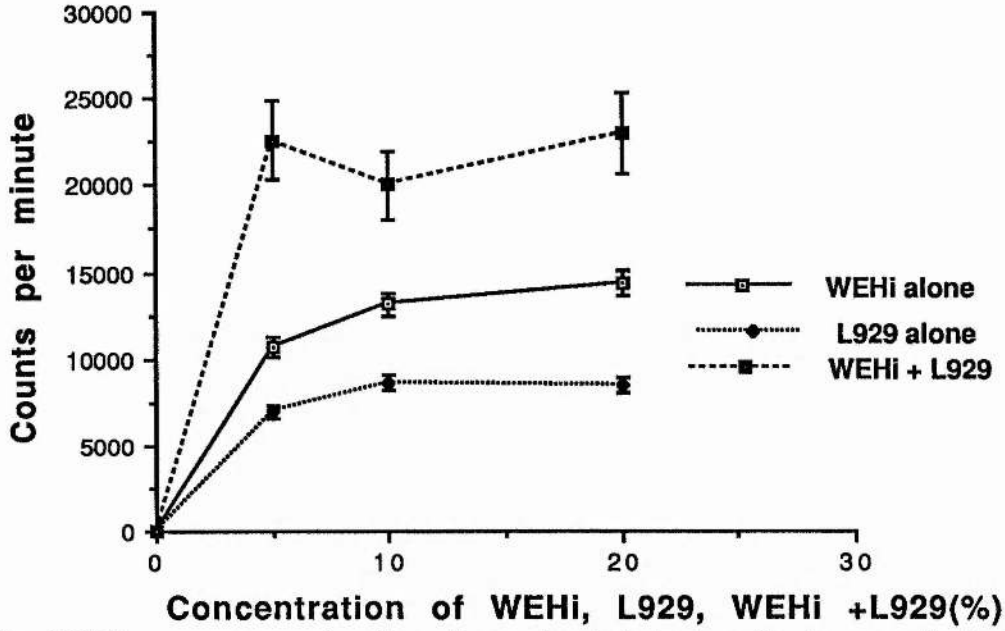


Fig.103:The response of mitoxantrone treated leuk cells to growth factors. SA7HDLeuk mice received mitox (0.75mg/Kg) 48hrs before they were moribound Growth factor response was determined using(3H)-thymidine uptake assay.

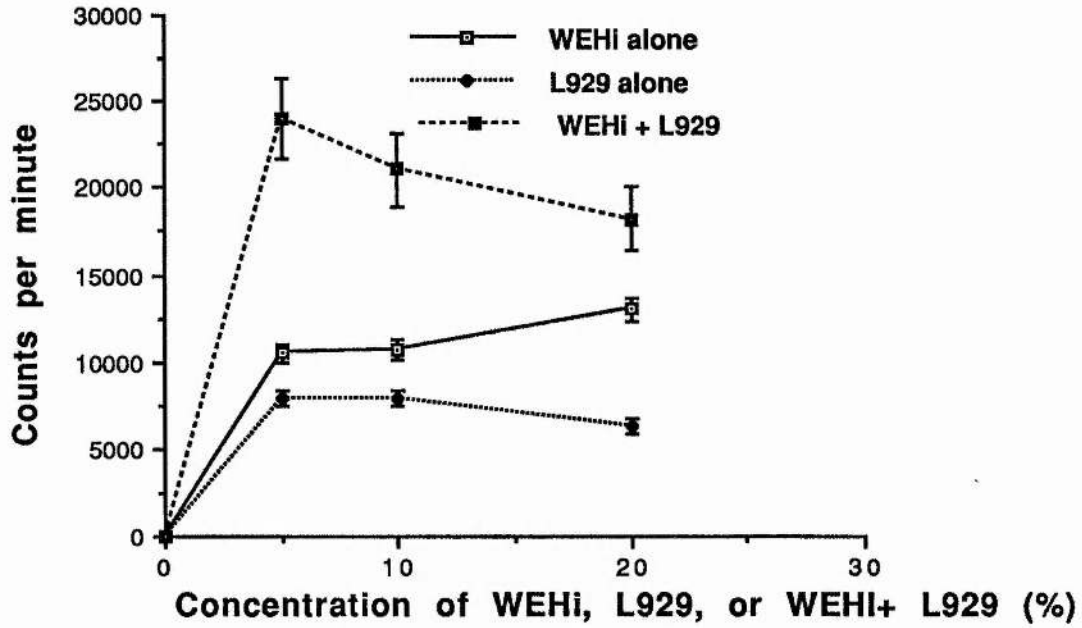


Fig.104: The response of (in vivo)mitox treated NBM cells to growth factors. Normal mice were treated with mitox(0.75mg/Kg) in vivo for 48hrs. Growth factor response was monitored using (3H)-thymidine uptake assay.

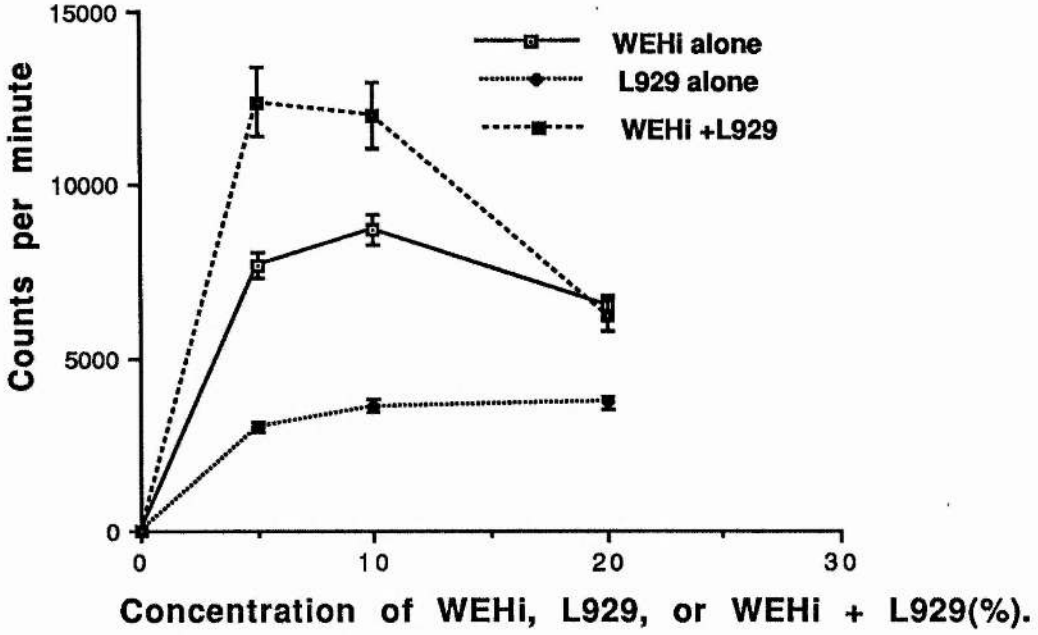


Fig.105: The response of untreated NBM cells to growth factors. Growth factor response was monitored using(3H)-TdR uptake assay.

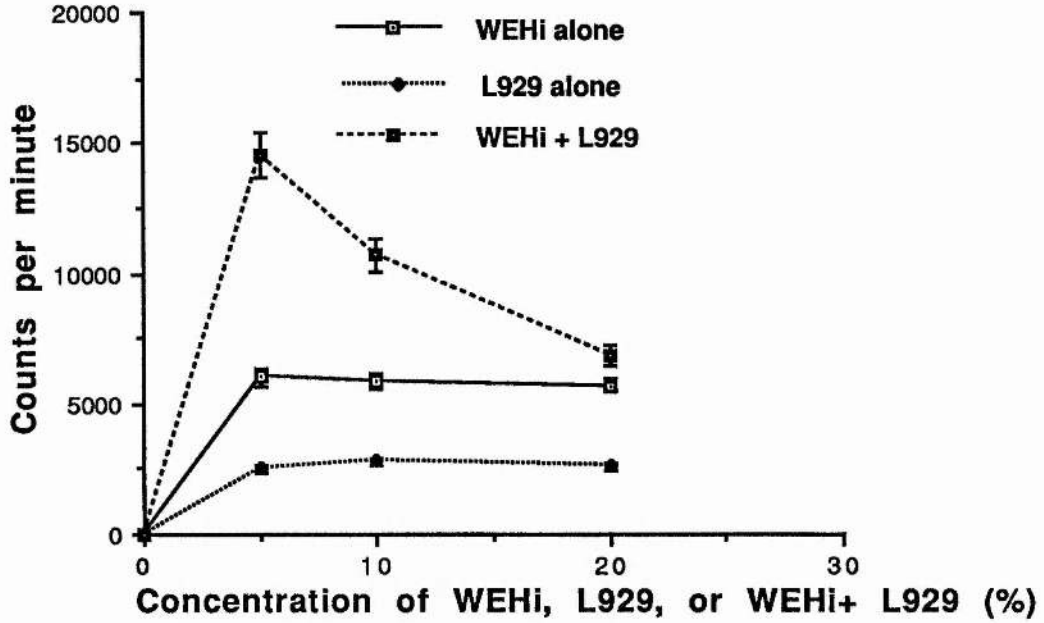


Fig.106:The response of (in vivo) mitox treated NBM cells to growth factors. Normal mice were treated with mitox(0.4mg/kg) 2days before autopsy. Growth factor response was monitored using (3H)-thymidine uptake assay.

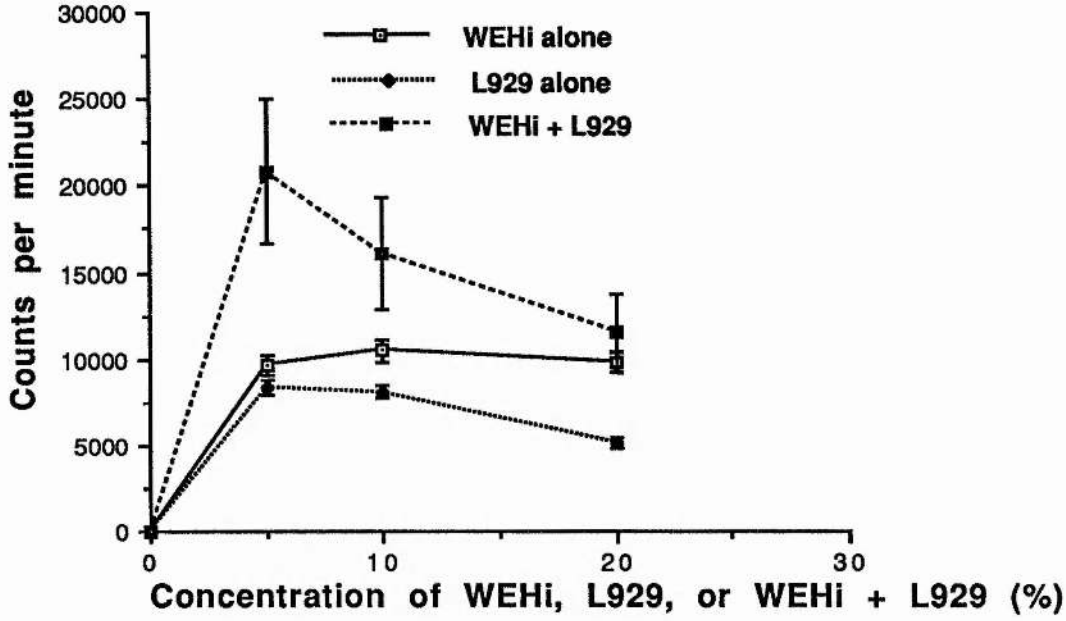


Fig.107: The responses of (in vivo)mitox treated NBM cells to growth factors. Normal mice were treated with mitoxantrone(2mg/Kg) , 9days before autopsy. Growth factor responses were monitored using (3H)-thymidine uptake assay.

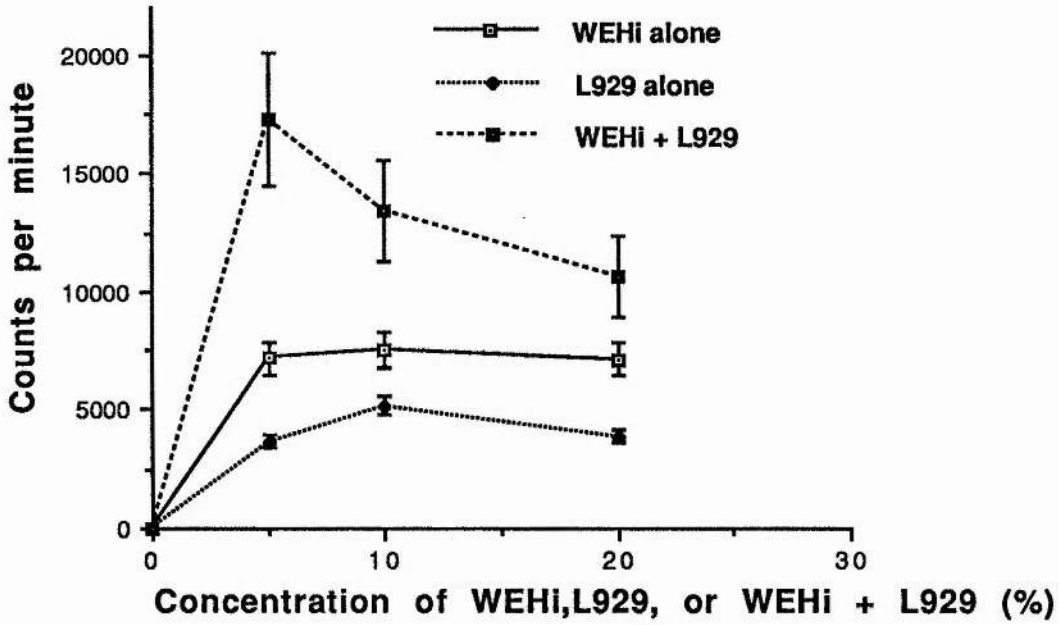


Fig.108: The responses of in vivo mitox treated NBM cells to growth factors. Normal mice were treated with mitox(3mg/Kg) and autopsied 9 days later. Growth factor responses were monitored using (3H)-thymidine uptake assay.

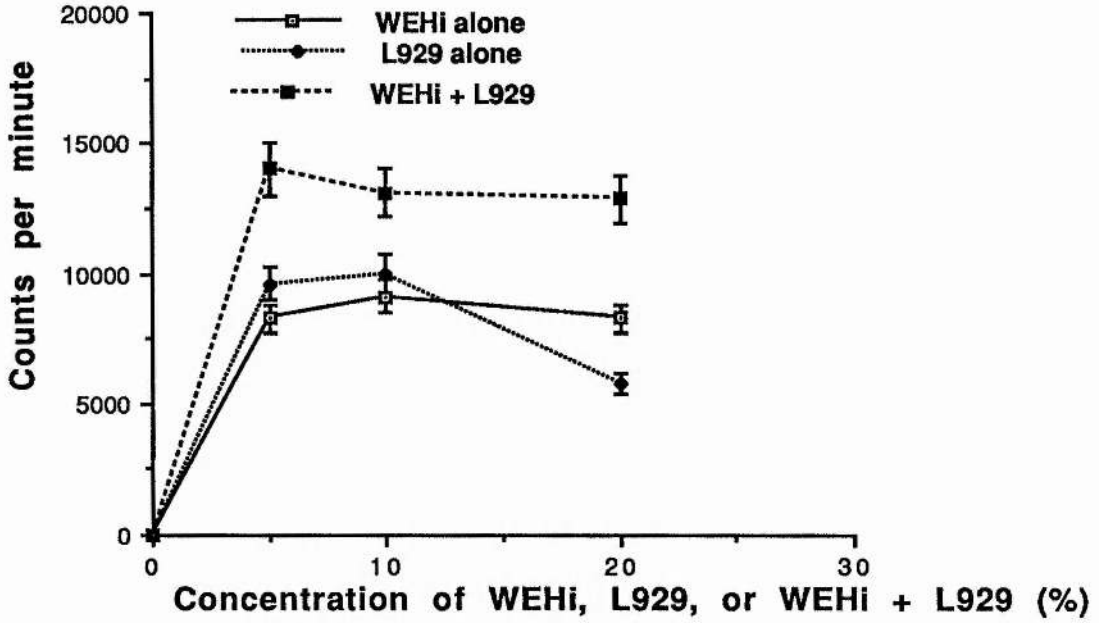


Fig.109: The resp of BM cells of mice dying of drug toxicity to growth factors. Mice were autopsied 30days after the initiation of treatment with mitox. No evidence of leukaemic infiltration of the spleen was observed.

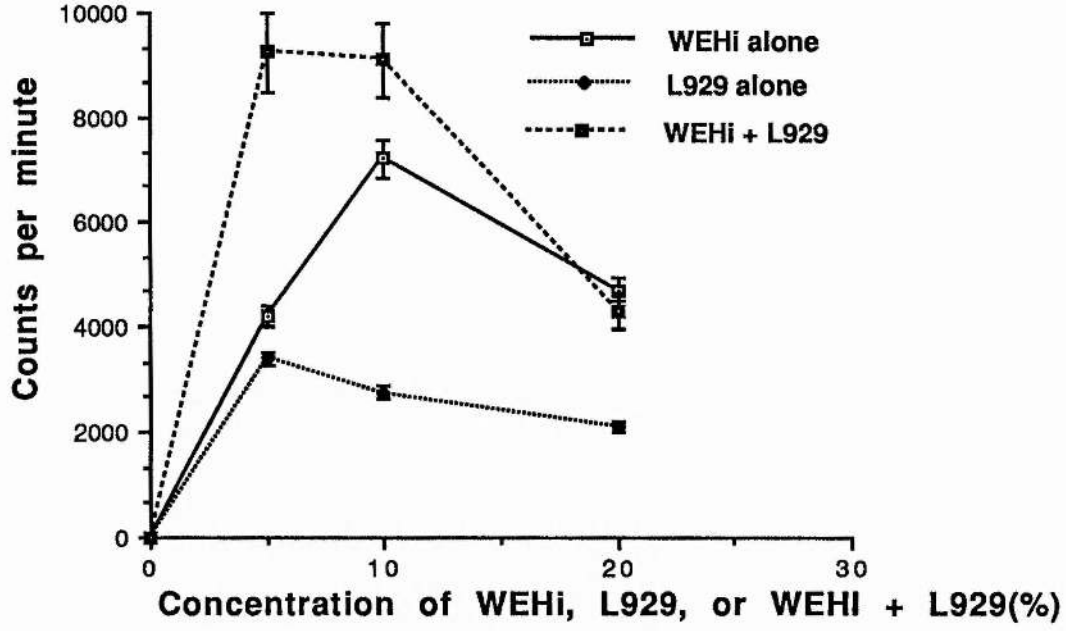


Fig.110: The response of BM cells from cured mice to growth factors. Mice bearing SA7HD leukaemia were treated with mitox(0.75mg/Kg) . Growth factor response was monitored using (3H)-thymidine uptake assay.

5.2.6 Growth Factor Response of Bone Marrow Cells of Cured Mice: Bone marrow cells of cured mice from both high cell dose transplant (Figures _110 and 111) and low cell dose transplant (Figure 112) including 90 day relapse (Figure 113) were responsive to growth factors. Similar results were obtained using bone marrow cells of normal mice treated with two doses of mitoxantrone 60 days previously (Figure 114). Thus, in all the doses and schedules studied, bone marrow cells of mitoxantrone treated normal mice always responded to growth factors. These data strongly suggests that only leukaemic cells became growth factor insensitive as a result of chemotherapeutic intervention with mitoxantrone.

5.2.7 Effect of Co-Culturing Recurrent Leukaemic Cells with NBM Cells: Recurrent leukaemic cells did not apparently produce factor(s) inhibitory to the growth of normal bone marrow cells in vitro. This is deduced from the observation that coculturing recurrent leukaemic cells with normal bone marrow cells did not inhibit the proliferative response of normal bone marrow cells to WEHi (Figure 115), L929 (Figure 116) and combinations of WEHi and L929 (Figure 117).

5.2.8 Colony Growth of Recurrent (SA7HD) Leukaemic Bone Marrow Cells: Very few colonies (6-7) were formed by recurrent leukaemic cells in agar culture. This is in contrast to high colony numbers (71±2) that were formed by untreated leukaemic cells (Table 9).

5.2.9 Recovery of Growth Factor Sensitivity and Colony Growth: Recovery of growth factor sensitivity was observed when recurrent leukaemic cells were passaged in normal mice. Recovery of growth factor

Table 9 : Number of colonies formed by $5x10^4$ recurrent leukaemic bone marrow cells. Recurrent leukaemic cells were either passaged in normal (untreated) mice or in mice pretreated with single or two doses of mitoxantrone (2 or 5 days prior to the transplant respectively)

	Number of Colonies		
Dose of Mitoxantrone (mg/kg)	*Recurrent leukaemic cells*	*First passage (TR1)*	*Second passage (TR2)*
0.75	6.0±0[a]	23±3[a]	30.0±5[a]
1.5mg/kg	7±2	22.0±5	68.0±14
PASS MIT 2d[b]	2.0±1	47.0±0	423.0±3
PASS MIT 50d[c]	3.0±0	50.0±1.	33.0±6
	29.0±2[d]	ND[e]	ND

Untreated leukaemic bone marrow cells gave 71+2 colonies/$5x10^4$ cells.

a. Mean ± SE of three separate experiments.

b. Recurrent leukaemic cells passaged in mice that received one dose (0.75mg/kg-I.P) of mitoxantrone two days previously.

c. Recurrent leukaemic cells were passaged in mice that received two doses (0.75mg/kg-IP) of mitoxantrone 50 days previously.

d. Leukaemic cells from one mouse responded to growth factors.

e. ND = not determined.

sensitivity was more pronounced (higher C.P.M) for recurrent leukaemia following treatment with mitox 0.4mg/Kg followed by 1.5mg/Kg and then 0.75mg/Kg which suggests that recovery was not dependent on initial mitoxantrone dose. Figures 118, 119 and 120 show the recovery of sensitivity to growth factors by recurrent leukaemic cells as a result of treating SA7 high cell dose transplant cell line (I.P) with 0.4, 0.75 and 1.5mg/Kg mitoxantrone (I.P)(given the code 0.4mg/KgTR1, 0.75mg/KgTR1 , 1.5mg/KgTR1) respectively. Each figure was the mean of 2-3 experiments. It was apparent that there was considerable variation (between experiments) in the recovery of growth factor sensitivity.

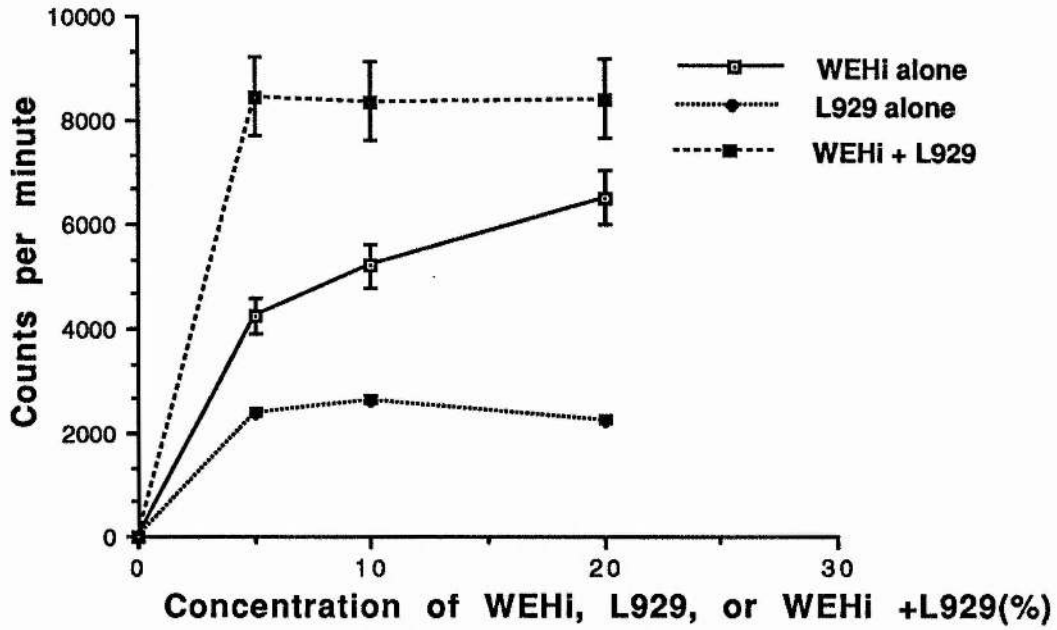


Fig.111: The response of BM cells from cured mice to growth factors. Mice bearing SA7HD leukaemia were treated with mitoxantrone(2mg/kg). Growth factor response was monitored using (3H)-thymidine uptake assay.

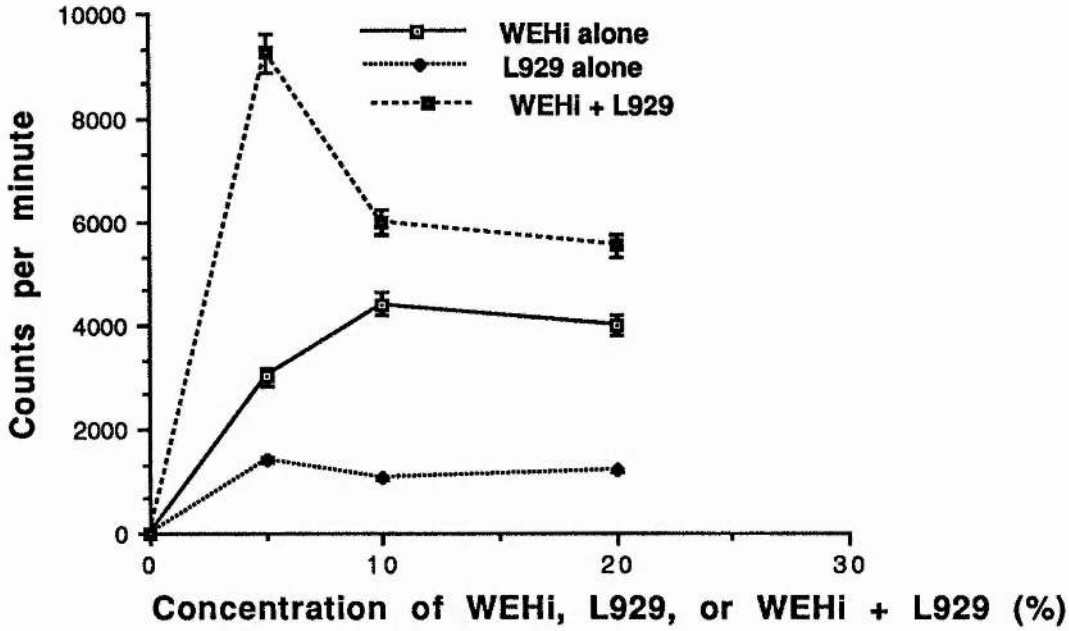


Fig.112: The response of BM cells of cured mice to growth factors. Mice bearing SA7LD leukaemia were treated with mitoxantrone(1.5mg/Kg). Growth factor response was monitored using (3H)-thymidine uptake assay.

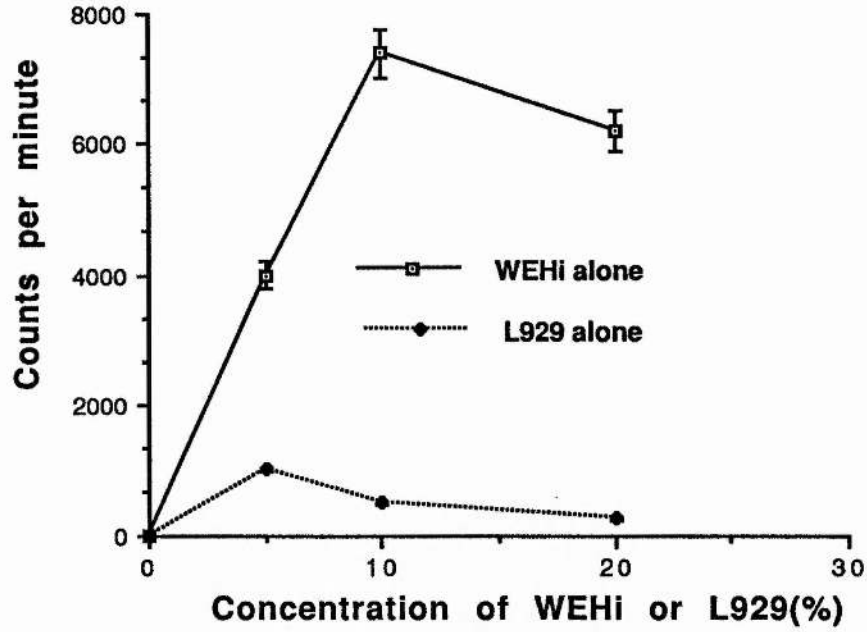


Fig.113: The resp of BM cells of mice with relapsed leuk to growth factors. Mice bearing the SA7LD leukaemia relapsed 90days following treatment. Growth factor response was monitored using (3H)-TdR uptake assay.

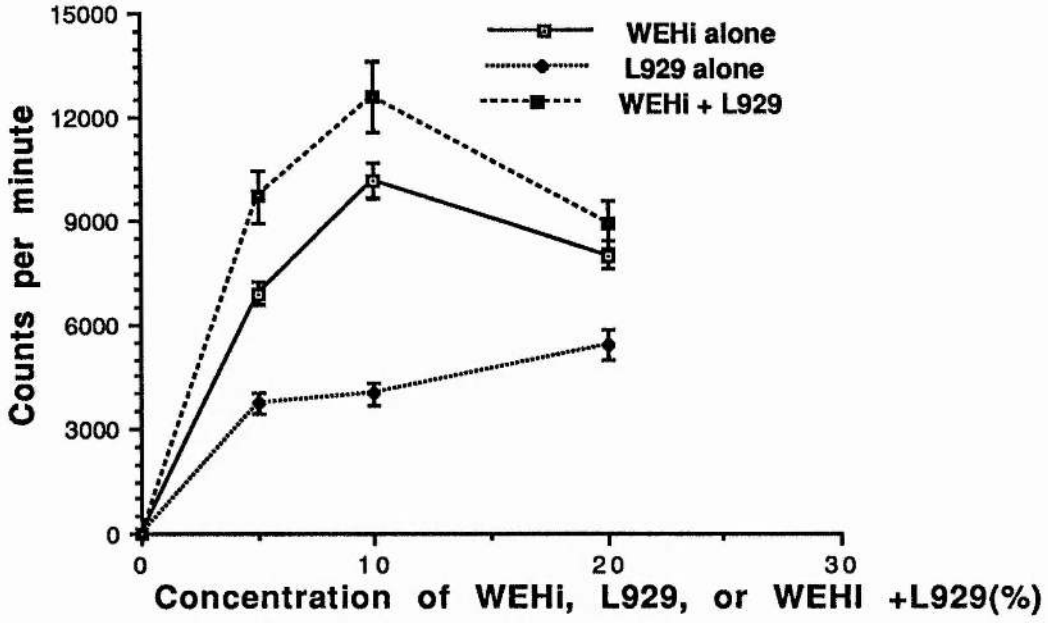


Fig.114: The response of (in vivo)mitox treated NBM cells to growth factors. Normal mice recieved mitox(0.75mg/Kg) and were autopsied 60 days later. Growth factor response was monitored using (3H)-thymidine uptake assay.

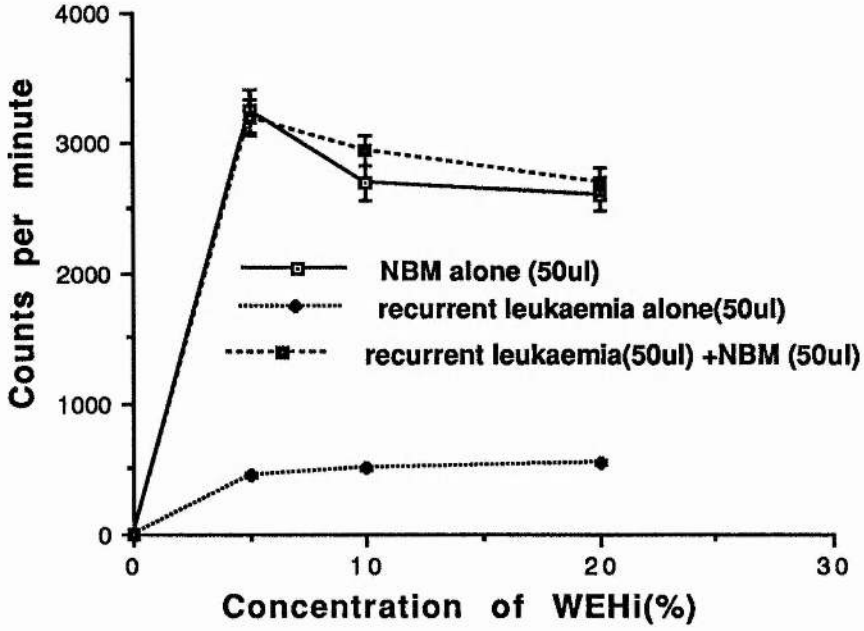


Fig.115: The resp of a mixture of NBM cells and rec leuk cells to WEHI. Growth factor response was monitored using (3H)-TdR uptake assay.

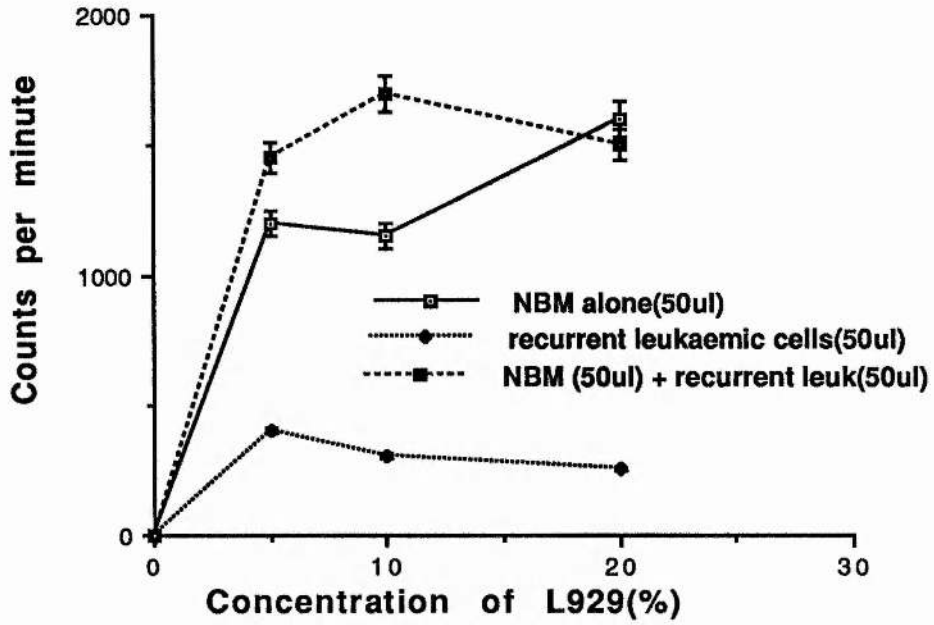


Fig116: The response of a mixture of NBM cells and rec leuk cells to L929. Growth factor response was monitored using (3H)-thymidine uptake assay.

Similarly, in an experiment monitoring the recovery of recurrent leukaemia as a result of treating 10^5 leukaemic cells with mitoxantrone 1.5mg/kg , the cells did not regain their sensitivity to growth factors to pretreatment levels even after one passage in normal mice (Figure 121). In the latter case, the leukaemic cells were not very responsive even to combinations of WEHi and L929. This suggests that the initial leukaemic load may also be important. Similar to what was observed in the [3H]TdR uptake assay, there was increased colony numbers in response to WEHi after recurrent leukaemic cells were passaged once in normal mice (Table 9).

5.2.10 Growth Factor Sensitivity and Colony Growth of Recurrent Leukaemia After Two Passages in Mice: After the 2nd passage of recurrent leukaemic cells in normal mice, there was increased proliferative response to single and combinations of growth factors. In this case too, recurrent leukaemic cells as a result of treatment with

mitoxantrone 1.5mg/Kg (given the code 1.5mg/KgTR2) were more sensitive to WEHi , L929 but not combinations of the two (Figure 122) as compared to recurrent leukaemia following treatment with mitoxantrone 0.75mg/Kg (Figure 123). Similarly, colony numbers following a second transplant were higher than those seen after the first transplant (Table 9). Interestingly, colony numbers after the second transplant were higher for 1.5mg/Kg TR2 as compared to 0.75mg/KgTR2 (Table 9) which suggest further agreement between colony assay and [3H]-thymidine uptake assay.

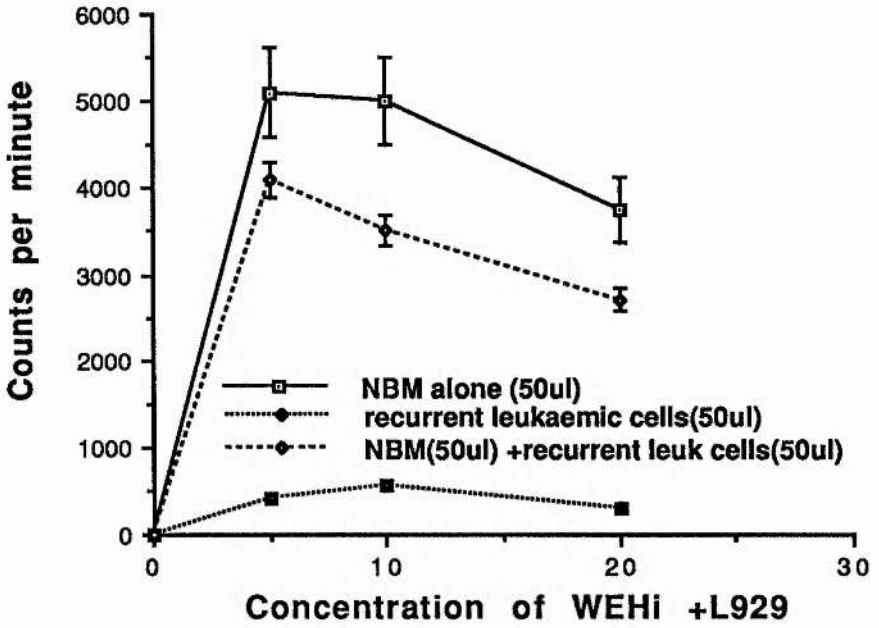


Fig.117: The resp of a mixture of NBM and recurrent leukaemia to WEHi +L929. Growth factor response was monitored using (3H) -TdR uptake assay.

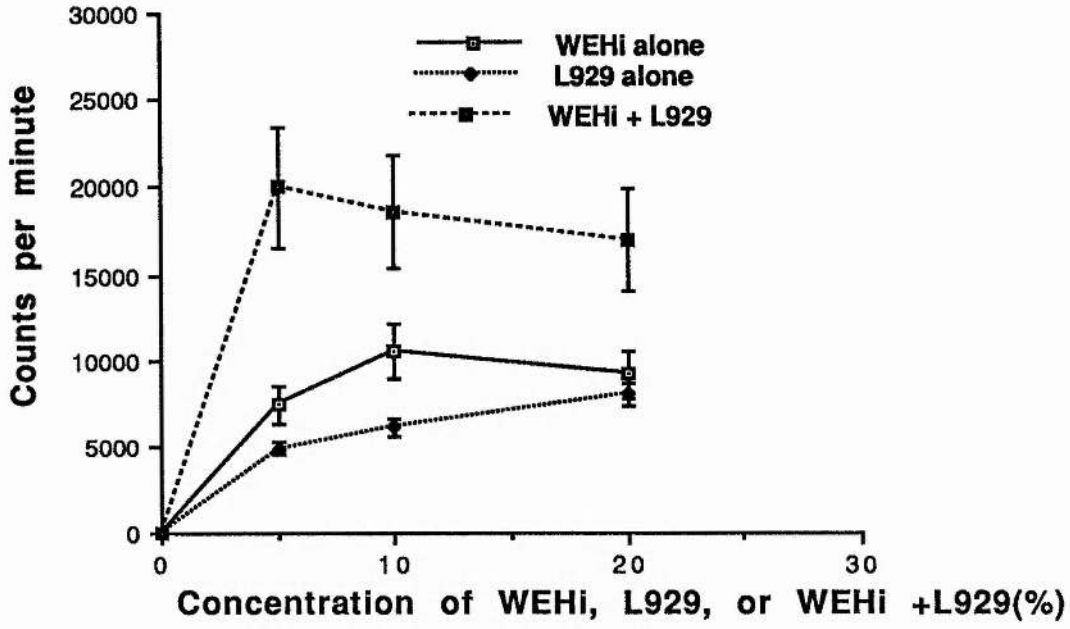


Fig.118: The response of rec. leuk. cells (0.4mg/KgTR1)to growth factors. The recurrent leukaemia was passaged once in norm mice. Growth factor response was monitored using (3H)-thymidine uptake assay.

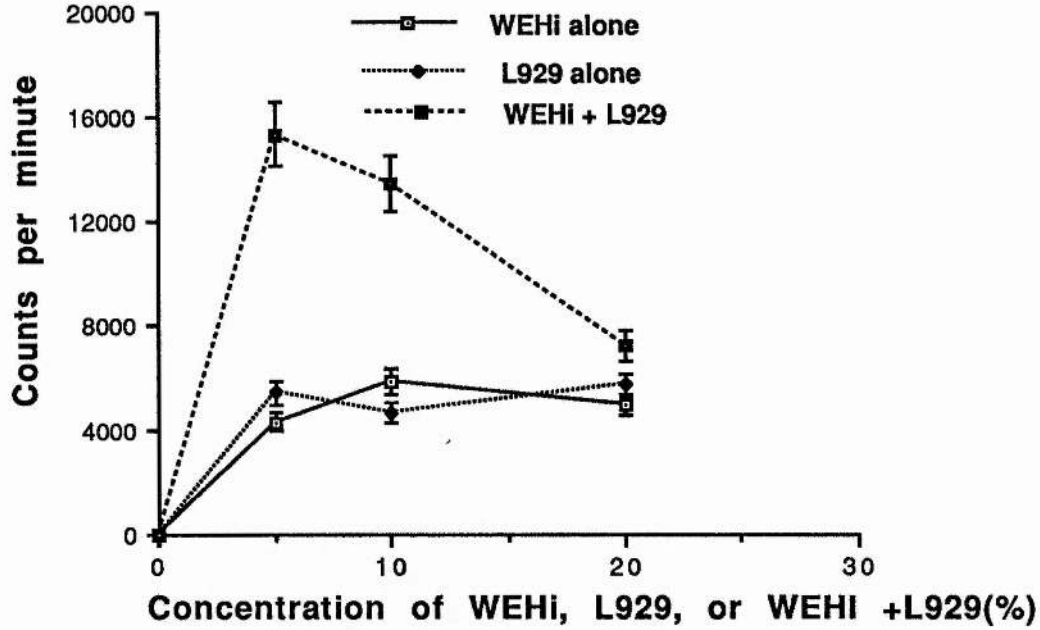


Fig.119: The response of rec.leuk.(0.75mg/KgTR1)to growth factors. The recurrent leukaemia was passaged once in norm. mice. Growth factor response was monitored using (3H)-TdR uptake assay.

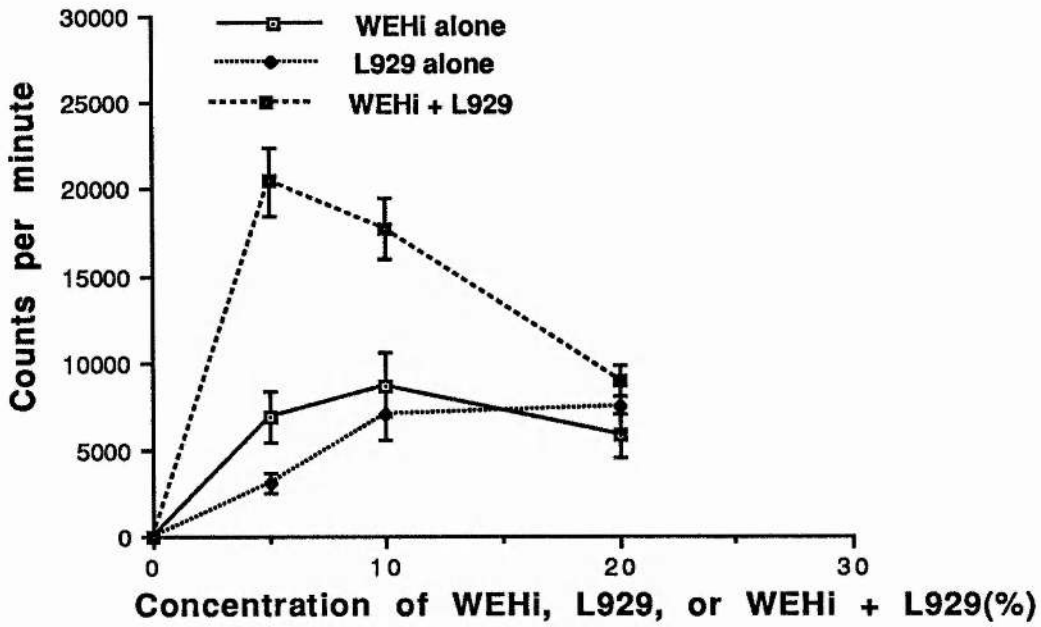


Fig.120:The response of rec.leuk. (1.5mg/KgTR1)to growth factors. The recurrent leukaemia was passaged once in normal mice Growth factor response was monitored using (3H)-thymidine uptake assay.

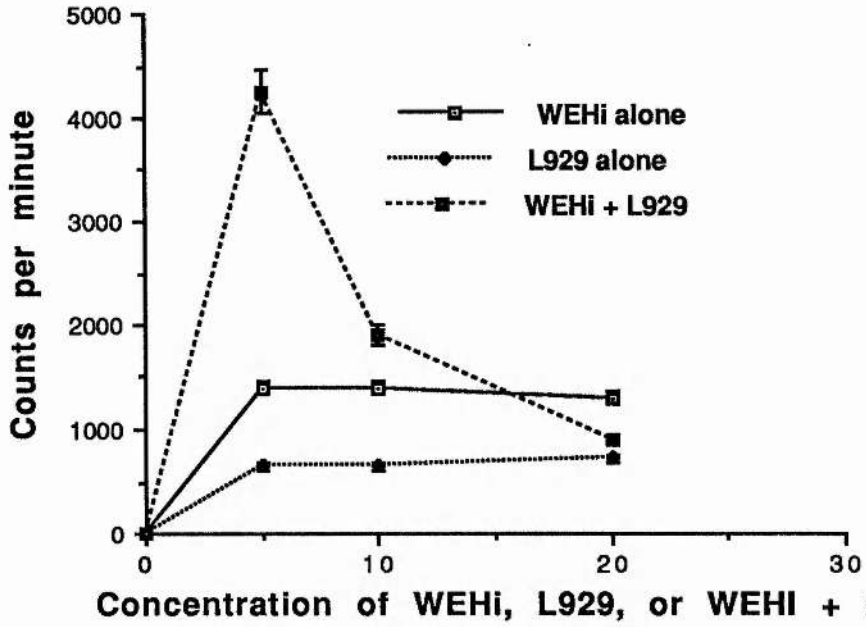


Fig.121: The response of recurrent leukaemia to growth factors.
The recurrent leukaemia (10 cells,i.p) was passaged once in norm mice. Growth factor response was monitored using (3H)-thymidine uptake assay.

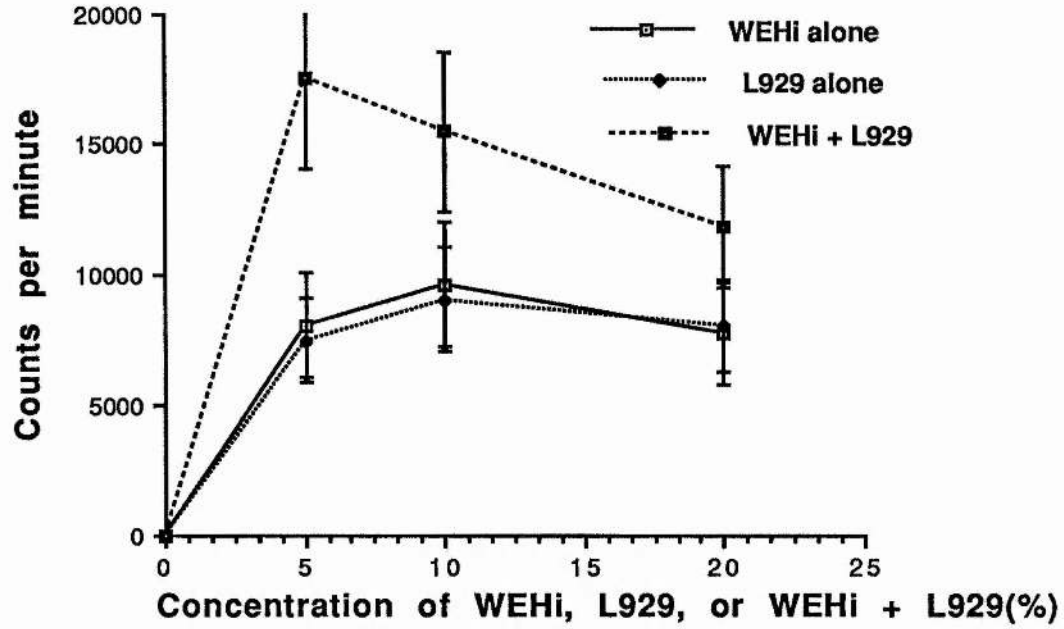


Fig.122: The response of rec.leuk.(1.5mg/KgTR2) to growth factors.
The recurrent leukaemia was passaged twice in normal mice
Growth factor response was monitored using (3H)-thymidine uptake assay.

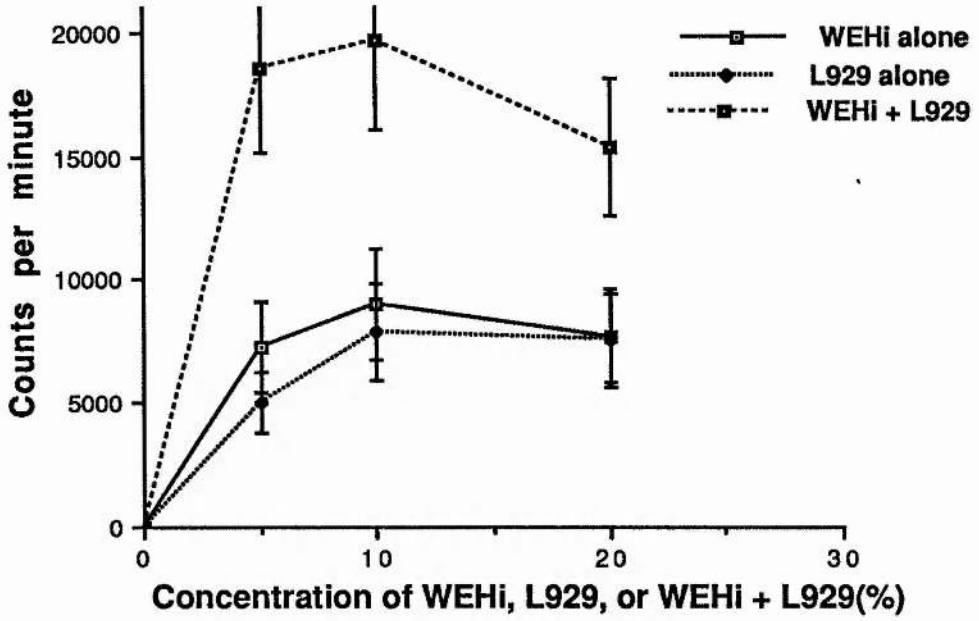


Fig.123:The response of rec leukaemic cells(0.75mg/Kg TR2) to growth factors.Growth factor response monitored using (3H)-thymidine uptake assay. The recurrent leukaemia was passaged twice in normal mice.

5.2.11 Effect of Passaging Recurrent Leukaemic Cells in Mice Pretreated with Mitoxantrone Two Days Previously: When recurrent leukaemic cells were passaged in mice that were pretreated with one dose of mitoxantrone (0.75mg/kg) two days previously (given the code PASS MIT 2d) (Figure 95) the leukaemia that subsequently developed (hereafter called second recurrent leukaemia) was insensitive to growth factors (WEHi, L929, WEHI+L929) in vitro. Figure 124 shows the response of such leukaemic cells to WEHi, L929 and combinations of the two (mean of two experiments). The leukaemia was insensitive to single or combinations of growth factors.

5.2.12 Effect of Passaging Second Recurrent Leukaemia(PASS MIT 2d) in Normal (untreated) Mice: When second recurrent leukaemia (PASS MIT 2d) was passaged in normal (untreated) mice (Figure 95) the resulting leukaemia (given the code PASS MIT 2dTR1) was responsive

to growth factors. Figure 125 shows the recovery of sensitivity to WEHi, L929 and combinations of WEHi and L929 by PASS MIT 2dTR1 cells. Each curve in the figure is the mean of three experiments.

5.2.13 Effect of Passaging PASS MIT 2d on In Vitro Colony Numbers: Only 2±1.0 colonies were formed when 5 x 10^4 PASS MIT 2d bone marrow(BM) cells were plated in agar (Table 9). However, when the leukaemic cells were passaged in normal (untreated) mice, there was a corresponding increase in colony numbers (47±4) thus agreeing with recovery of growth factor sensitivity monitored using (3H)-thymidine uptake assay reported above.

5.2.14 Effect of Passaging PASS MIT 2dTR1 in Normal (untreated) Mice: The sensitivity of leukaemic bone marrow cells to WEHi, L929 and combinations of WEHi+L929 after passaging PASS MIT 2dTR1 in normal (untreated) mice was monitored. The leukaemic cells (now given the code PASS MIT 2d TR2) were as responsive to growth factors as PASS MIT 2dTR1 (Figure 126).

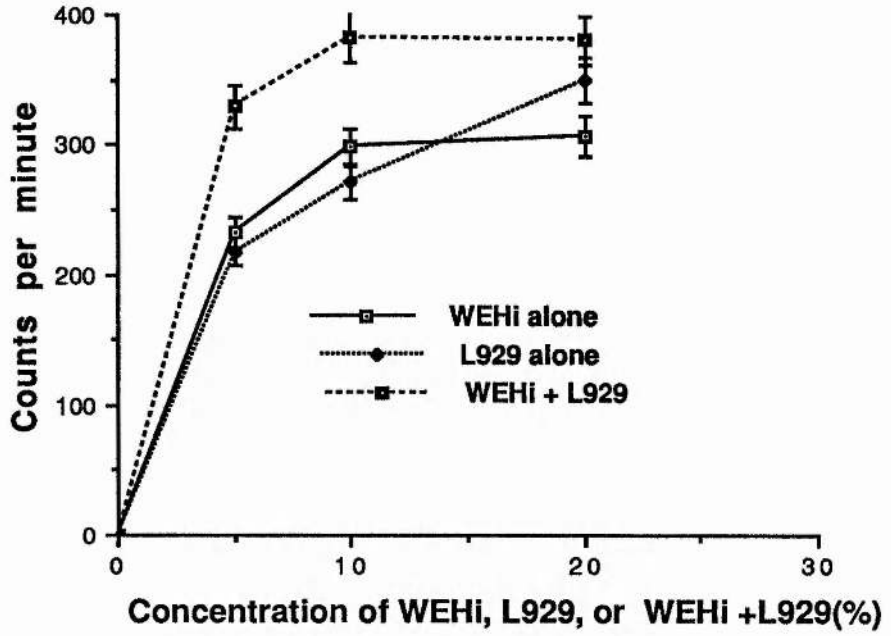


Fig. 124: The response of recurrent leukaemia(PASS MIT 2d) to growth factors. Growth factor responses monitored using(3H)-thymidine uptake assay. The recurrent leukaemia was passaged in mitox. pretreated mice.

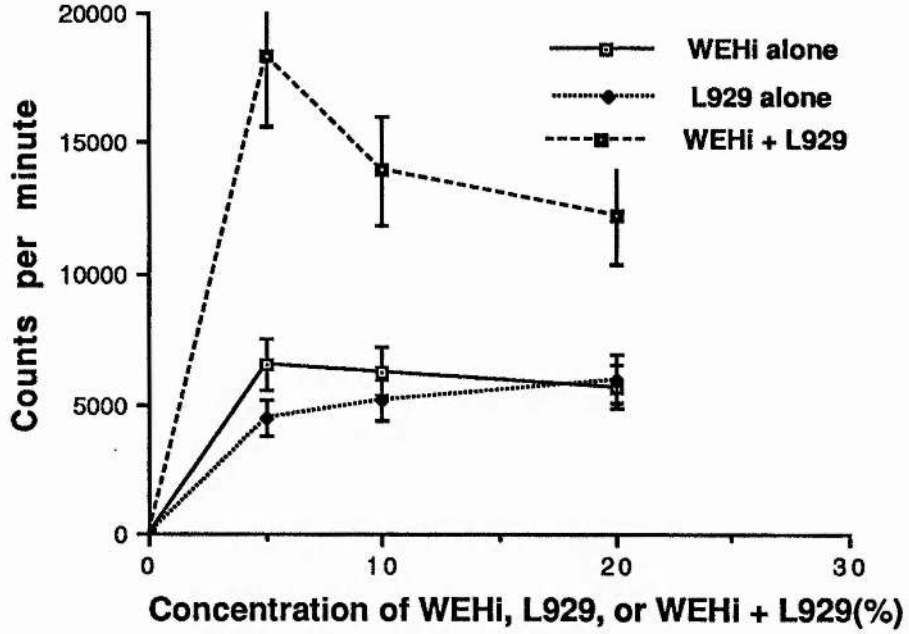


Fig.125: The response of PASS MIT 2dTR1 to growth factors. Growth factor response was monitored using (3H)-thymidine uptake assay. The recurrent leukaemia was passaged once in normal mice.

5.2.15 Effect of Passaging PASS MIT 2dTR1 on Colony Numbers: PASS MIT 2dTR1 was further passaged in normal mice, and the resulting leukaemia (PASS MIT 2d TR2) was plated in agar. A decrease in colony numbers was observed as compared to PASS MIT 2dTR1 (Table 9). This contrasts with the no difference in growth factor sensitivity observed between PASS MIT 2dTR1 and PASS MIT 2d TR2 when response was monitored using (3H)-thymidine uptake assay.

5.2.16 Effect of Passaging Recurrent leukaemia in Mice Pretreated with Two Doses of Mitoxantrone (0.75mg/kg) 50 days previously: Recurrent leukaemic cells were passaged in three mice (M_1, M_2 and M_3) that were pretreated 50 days previously with two doses of mitoxantrone (0.75mg/Kg). The growth factor sensitivity of leukaemic bone marrow cells of these mice was monitored in vitro. The leukaemic cells (given the code PASS MIT 50d) were insensitive to growth factors in 2 of 3 mice. Figure 127 shows the response of PASS MIT 50d to WEHi, L929 and combinations of the two. Whereas, bone marrow cells of M_1 and M_2 did not respond to these growth factors, those of M_3 did respond.

5.2.17 Colony Assay of PASS MIT 50d: Whereas leukaemic bone marrow cells of M_1 and M_2 formed 3.6±0.2 colonies when plated in agar, those of M_3 formed 29±2 colonies (Table 9). This indicates further agreement between colony assay result and [3H]TdR uptake results.

5.2.18 Effect of Passaging PASS MIT 50d in Normal (untreated) Mice:

When growth factor insensitive PASS MIT 50d was passaged into normal (untreated) mice, (now given the code PASS MIT 50d TR1), its growth factor sensitivity was restored. The leukaemic cells became extremely sensitive to WEHi, L929 and combinations of the two (Figure 128). The figures were mean of three experiments.

5.2.19 Effect of Passaging PASS MIT 50d on In vitro Colony Growth:
Similar to what was observed with [3H]TdR uptake assay, passaging PASS MIT 50d in normal mice resulted in an increased colony formation by the leukaemic bone marrow cells (Table 9) (50±1 vs 3.6±0.2).

5.2.20 Effect of Passaging PASS MIT 50d TR1 on Growth Factor Sensitivity
and Colony Growth: When PASS MIT 50d TR1 was further passaged in normal (untreated) mice, the resulting leukaemia (given the code PASS MIT 50d TR2) was less responsive to WEHi, L929 or their combinations as compared to PASS MIT 50d TR1. Figure 131 shows the response of PASS MIT 50d TR2 to WEHi, L929 and combinations of the two. Each figure was the mean of three experiments.

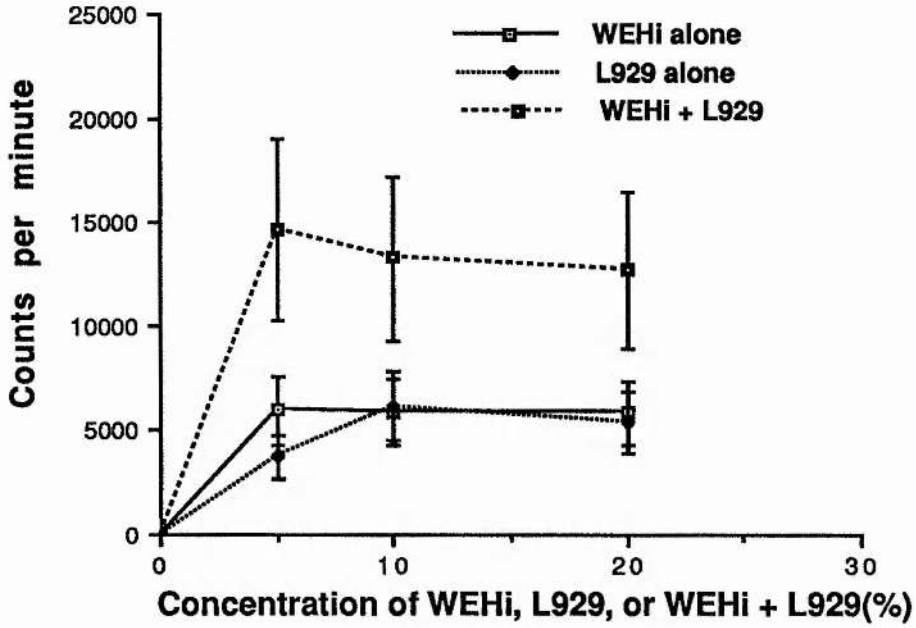


Fig. 126: The response of PASS MIT 2d TR2 to growth factors.
Growth factor response was monitored using(3H)-thymidine uptake assay.
The recurrent leukaemia was passaged twice in normal mice.

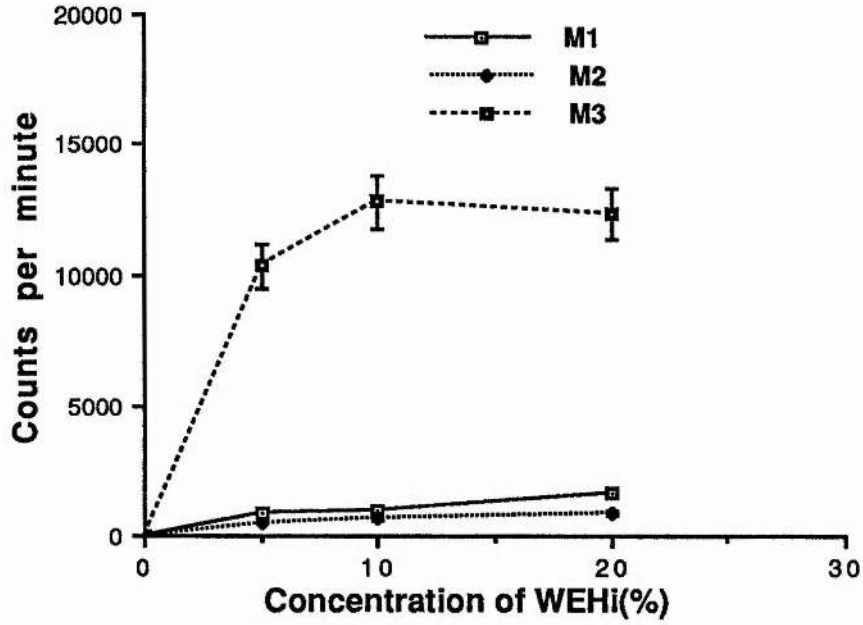


Fig.127: The response of PASS MIT 50d to WEHI.
M1,M2,and M3 represent three different mice.
Growth factor response was monitored using (3H)-TdR uptake assay.

The decrease in response to growth factors between PASS MIT 50d TR1 and PASS MIT 50d TR2 was also reflected by a fall in colony numbers between the two passages (Table 9). Although, sensitivity to growth factors was restored after a single passage of the recurrent leukaemic cells in normal (untreated) mice (according to [3H]TdR uptake assay results) , colony numbers (except probably for 1.5mg/KgTR1) did not return to pretreatment levels even after the second passage (Table 9).

5.2.21 Comparison of Recurrent Leukaemic Spleen Weights in Mitoxantrone Pretreated or Untreated Mice: Pooled spleen weights from mitoxantrone pretreated mice bearing recurrent leukaemia were up to 80mg heavier than comparable spleens from untreated normal mice. It is likely that the presence of mitoxantrone in the pretreated mice triggered the leukaemic cells to divide hence the increased spleen weight.

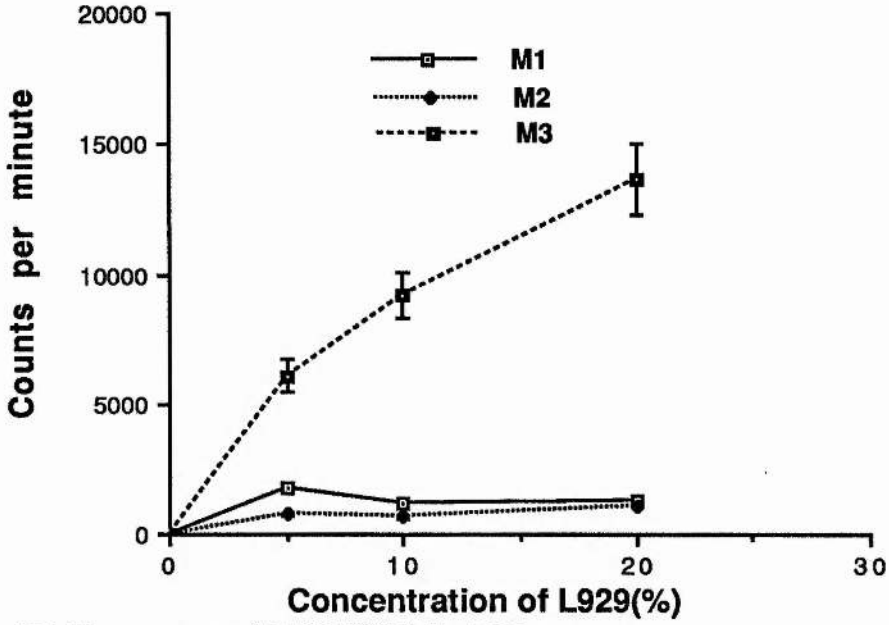


Fig.128: The response of PASS MIT 50d to L929.
M1, M2, and M3 represent three different mice.
Growth factor response was monitored using(3H)- TdR uptake assay.

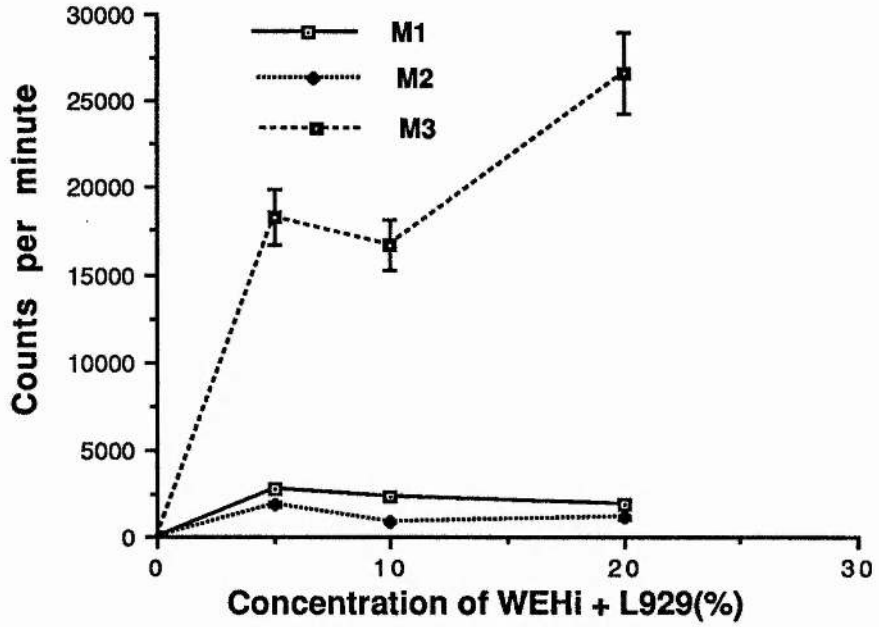


Fig.129: The response of PASS MIT 50d to WEHI + L929.
M1,M2, and M3 represent three different mice.
Growth factor response was monitored using (3H)-TdR uptake assay.

Comparison of In Vitro Colony Numbers Produced by Recurrent Leukaemic Bone Marrow Cells from Pretreated or Untreated Mice: Colony numbers formed by bone marrow cells of PASS MIT 2dTR1 and PASS MIT 50d TR1 were higher than those formed after the first passage of recurrent leukaemia in untreated normal mice (Table 9).

5.2.22 In Vitro Mitoxantrone Sensitivity of Recurrent Leukaemic Cells After

Passage in Normal (untreated) Mice: Even after two passages in normal mice, the sensitivity of recurrent leukaemic cells to subsequent mitoxantrone treatment in vitro did not revert to pretreatment levels. This resistance was dependent on the initial mitoxantrone dose the leukaemic cells were exposed to in vivo. Figure 132 shows the mitoxantrone dose-response curve of recurrent leukaemia following treatment of SA7 high cell dose leukaemic cells with 0.4mg/Kg mitoxantrone in vivo (0.4mg/Kg mitox) after it was passaged once in normal (untreated) mice (given the code 0.4mg/Kg TR1). The cells were even slightly more sensitive than untreated leukaemic cells to mitoxantrone (Figure 132). This is further shown by the following example: 0.4mg/Kg TR1 was as sensitive to Ara-C as untreated leukaemic cells (Figure 133). However, when mitoxantrone (1.2ng/ml) was added, the cells became more sensitive to the combination as compared to untreated leukaemic cells (Figure 133). 0.4mg/Kg TR1 cells became more sensitive to Ara-C treatment in vitro if a combination of WEHi (5%) and L929 (5%) was used to stimulate their proliferation in vitro (Figure 134). Similarly, combinations of Ara-C (0.12-120ng/ml) with mitoxantrone (1.2ng/ml) produced greater effects when a combination of growth factors rather than single growth factor (WEHi) was used to stimulate the in vitro proliferation of the cells (Figure 134).

Apparently mitoxantrone alone or in combination with Ara-C (1.2ng/ml) also seems more effective when combination of WEHi (5%) and L929 (5%) was used to stimulate the cells in vitro as compared to when WEHi alone was used (Figure 135).

When mitoxantrone 0.75mg/Kg was used in the treatment of SA7 high cell dose transplant leukaemia, the recurrent leukaemic cells (after two passages in normal untreated mice (0.75mg/Kg TR2) were less sensitive to low concentrations (0.12-1.2ng/ml) of mitoxantrone and stimulation of [3H]TdR uptake rather than inhibition of DNA synthesis was observed with these concentrations (Figure 136).

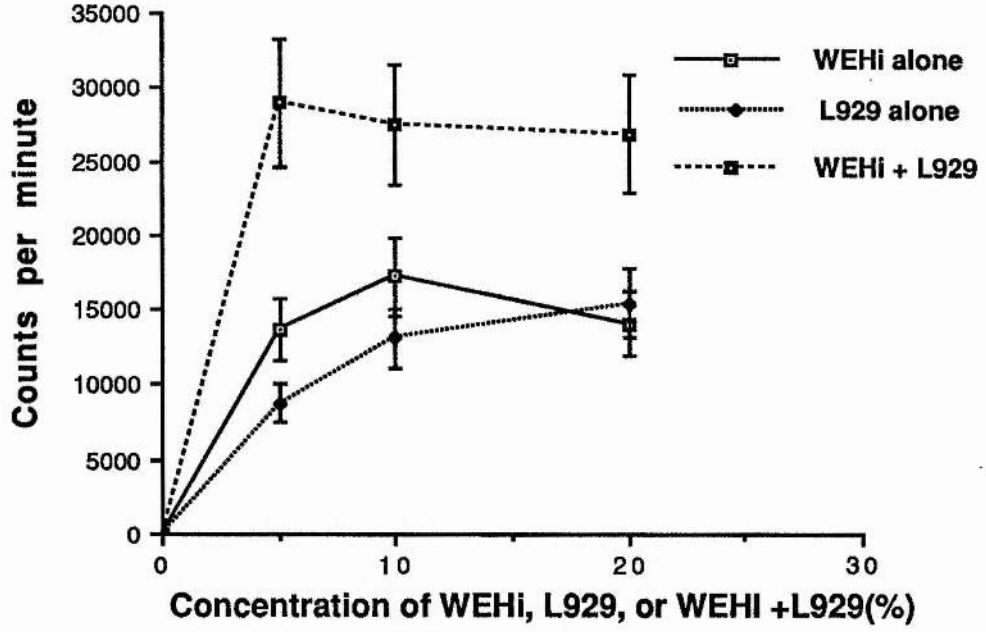


Fig.130: The response of PASS MIT 50dTR1 to growth factors.
Growth factor response was monitored using (3H)-TdR uptake assay.
The recurrent leukaemia was passaged once in normal mice.

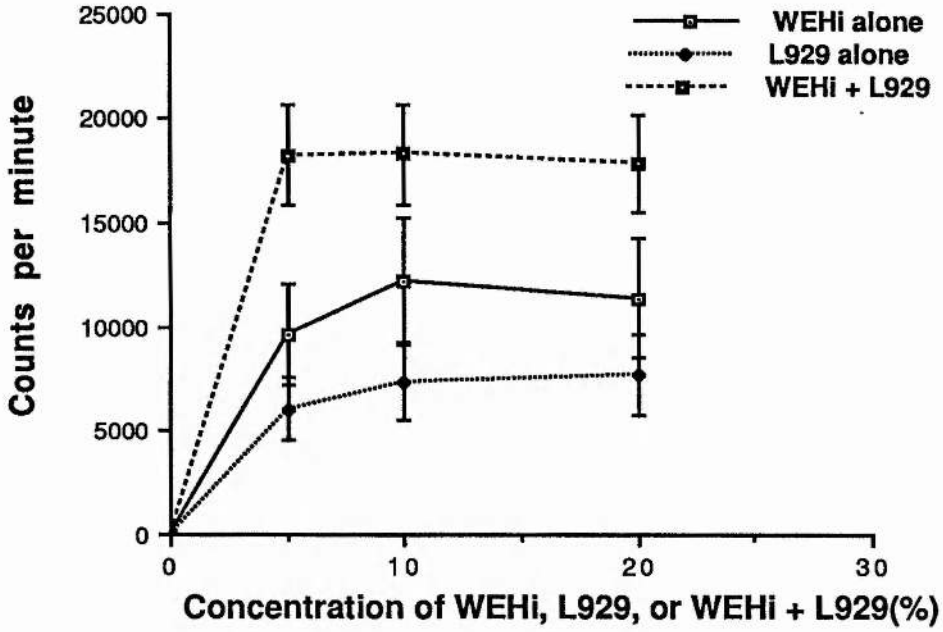


Fig.131: The response of PASS MIT 50d TR2 to growth factors.
Growth factor responses were monitored using (3H)-TdR uptake assay.
The recurrent leukaemia was passaged twice in normal mice.

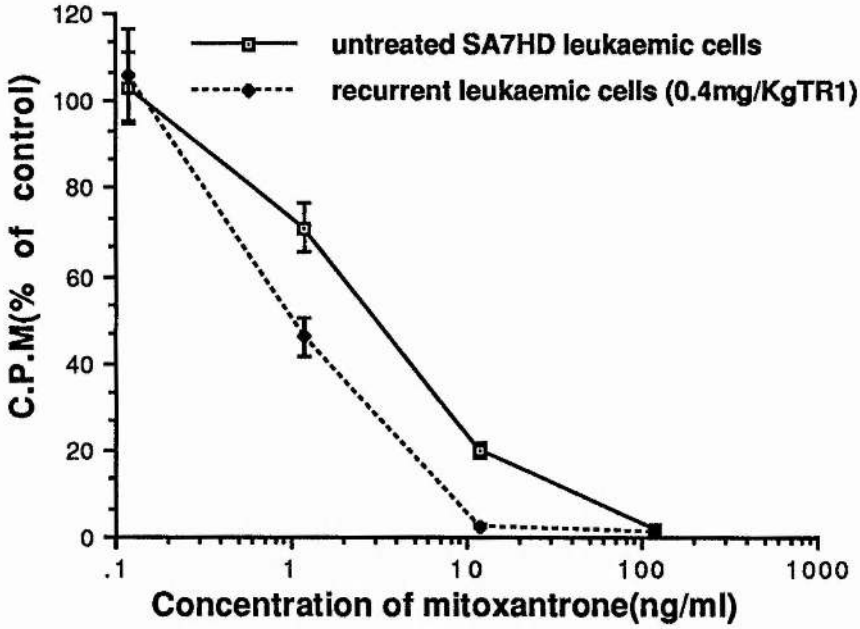


Fig.132: The response of rec leuk cells(0.4mg/Kg TR1) to mitoxantrone.
Cytotoxicity was determined using(3H)-thymidine uptake assay.

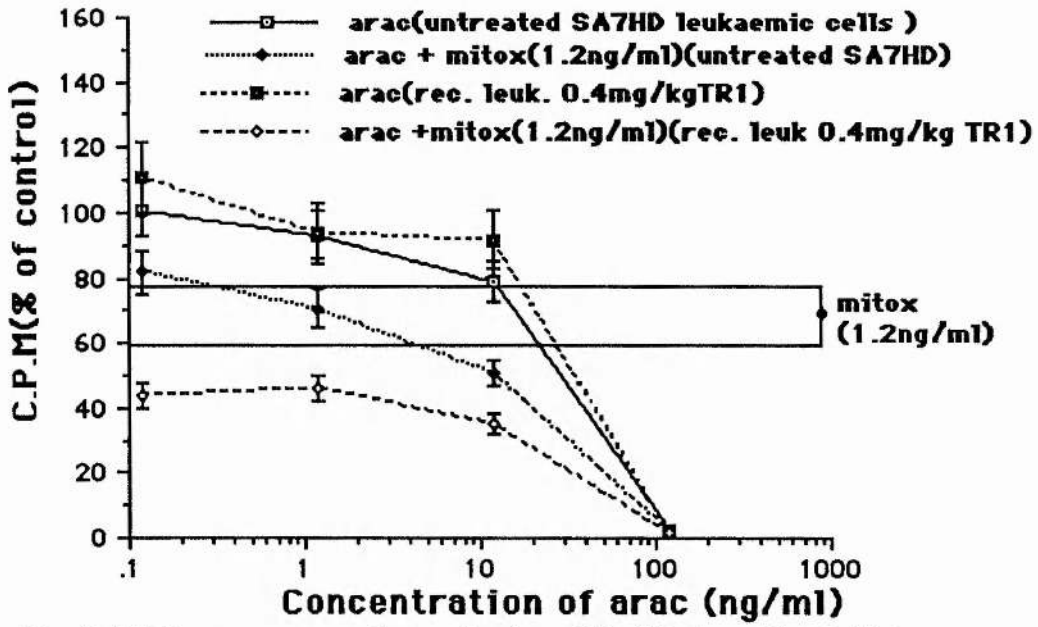


Fig.133:The response of rec. leuk. cells (0.4mg/KgTR1) to arac alone or in combination with mitoxantrone monitored in vitro. Cytotoxicity was determined using the (3H)-thymidine uptake assay

At higher mitoxantrone concentrations (12-120ng/ml) there was no difference in sensitivity between recurrent leukaemic cells (0.75mg/kg TR2) and untreated leukaemic cells. This is shown clearly in Figure 136, which is mean of 3 separate experiments.

Recurrent leukaemia following treatment of SA7HD with mitoxantrone (1.5mg/Kg) was markedly insensitive to subsequent mitoxantrone treatment in vitro even after the recurrent leukaemic cells were passaged twice in normal (untreated) mice (1.5mg/Kg TR2). Figure 137 shows the response (mean of 3 experiments) of 1.5mg/Kg TR2 to mitoxantrone treatment in vitro. A resistant factor ($I.C_{50}$ of mitoxantrone against 1.5mg/Kg TR2 divided by $I.C_{50}$ of the drug against untreated SA7HD leukaemic cells) of 17 was found. Whereas 12ng/ml of mitoxantrone inhibited [3H]TdR uptake by 80% (relative to control) in untreated SA7HD leukaemic cells (Figure 33), the same concentration stimulated [3H]TdR uptake (and hence proliferation?) of the recurrent leukaemic cells (1.5mg/kg TR2) to well above control values (138±22%)(Figure 137). If such stimulation of the recurrent leukaemic cells by mitoxantrone occurs in vivo, it could explain the increased spleen weights in mice pretreated with mitoxantrone.

These data strongly suggests that the degree of mitoxantrone resistance in vitro depended on the dose of mitoxantrone administered to the leukaemic cells in vivo. This is shown clearly in Figure 138 where there was increasing insensitivity to in vitro mitoxantrone (in the concentration range 0.12-12ng/ml) with increasing dose of in vivo administered mitoxantrone. The resistance developed by 1.5mg/Kg TR2 to in vitro treatment with mitoxantrone (within the concentration range 1.2-12ng/ml) was significantly different ($P=0.012$) from untreated leukaemic cells using the Mann-Whitney test.

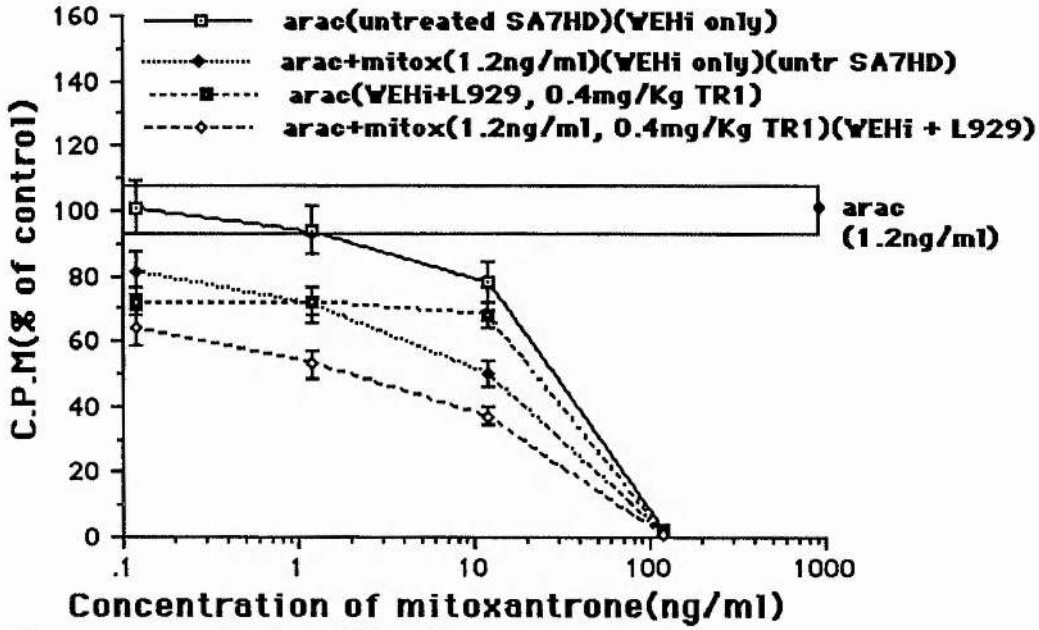


Fig.134: The response of 0.4mg/KgTR1 to arac alone or in combination with mitox. The recurrent leukaemic cells were stimulated by a combination of WEHi(5%) and L929(5%). The (3H)-thymidine uptake assay was used.

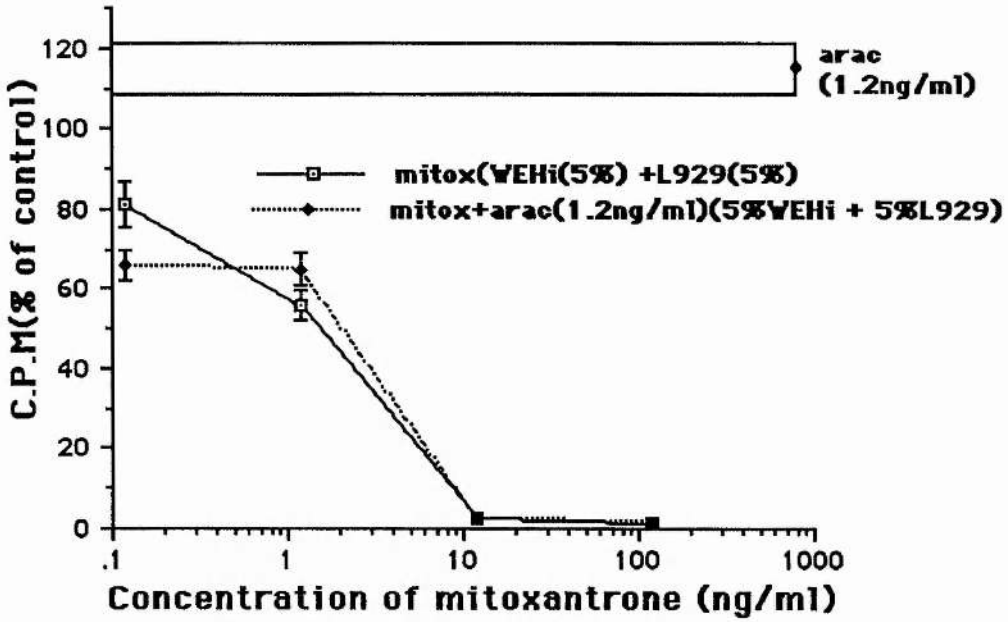


Fig.135: The response of 0.4mg/KgTR1 to mitoxantrone alone or in combination with arac monitored using the (3H)-thymidine uptake assay. The rec. leuk cells were stimulated by a combination of WEHi and L929.

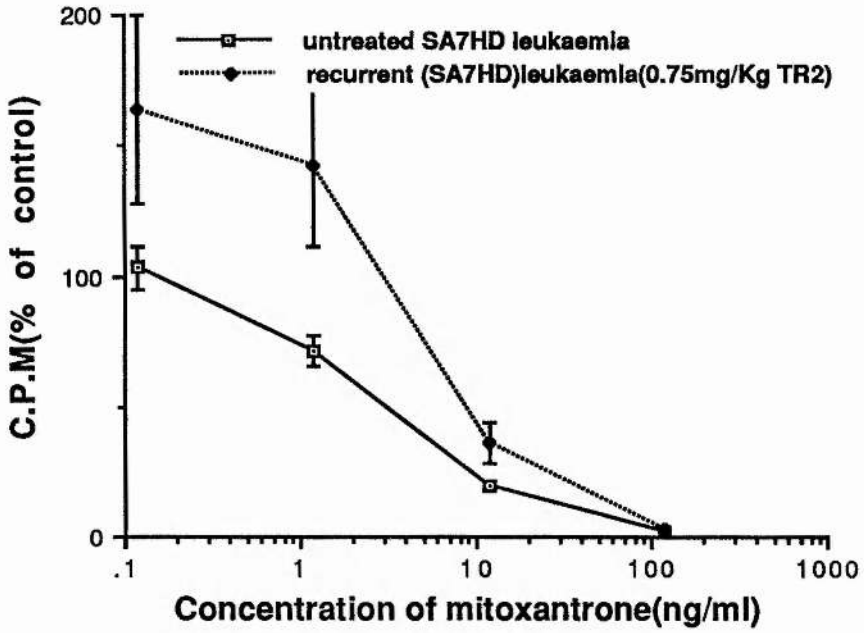


Fig.136: The response of rec leuk(0.75mg/Kg TR2) to mitoxantrone. Cytotoxicity was determined using(3H)-thymidine uptake assay in vitro. The rec. leuk. was passaged twice in normal mice.

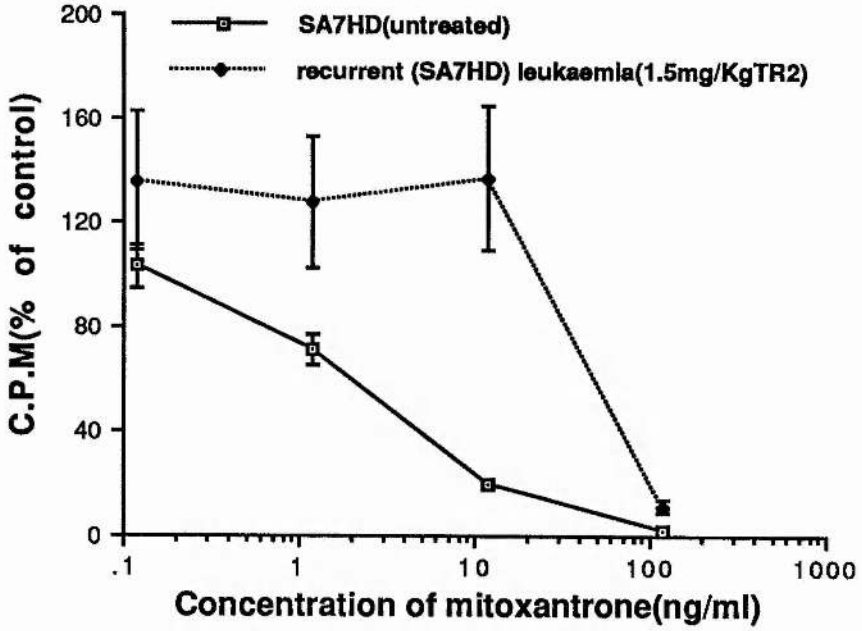


Fig.137: The response of rec.leuk.(1.5mg/KgTR2) to mitoxantrone. Cytotoxicity was determined using (3H)-thymidine uptake assay in vitro. The rec. lekaemia was passaged twice in normal mice.

5.2.23 In Vitro Mitoxantrone Sensitivity of Recurrent Leukaemic Cells After Passage in Mitoxantrone Pretreated Mice: Surprisingly, no increased resistance to mitoxantrone treatment in vitro was observed when recurrent leukaemic cells were first passaged in mitoxantrone pretreated mice before passaging again in untreated normal mice. This supports the view that it was the initial dose the leukaemic cells were first exposed to (in vivo), that determined the degree of any subsequent resistance they develop. Subsequent exposures after the first apparently had no effect. Figure 139 shows the response of PASS MIT 2dTR1 to mitoxantrone treatment in vitro (mean of 3 experiments). The recurrent leukaemic cells were less sensitive to mitoxantrone as compared to untreated leukaemic cells only at low concentration within the range 0.12-12ng/ml. However, with further passage in normal (untreated) mice (PASS MIT 2d TR2) there was no difference in sensitivity as

compared to untreated leukaemic cells (Figure 140) (mean of 3 experiments).

Figure 141 shows the response of PASS MIT 50d TR1 to in vitro treatment with mitoxantrone (mean of 2 experiments). No difference in sensitivity to mitoxantrone was observed. However, with further passage in normal (untreated) mice, PASS MIT 50d TR2 became less sensitive to low concentrations of mitoxantrone (0.12-1.2ng/ml) where stimulation rather than inhibition of [3H]TdR uptake was observed (Figure 142) (mean of 3 experiments). Thus, whereas PASS MIT 2dTR1 became more sensitive to mitoxantrone with passaging , PASS MIT 50TR1 on the other hand, became less sensitive to low concentrations of mitoxantrone after passaging. However, none of these recurrent leukaemias was as insensitive to mitoxantrone as 1.5mg/Kg TR2 thus, lending support to the observation that no increase in mitoxantrone insensitivity was seen when recurrent leukaemic cells were passaged in mitoxantrone pretreated mice.

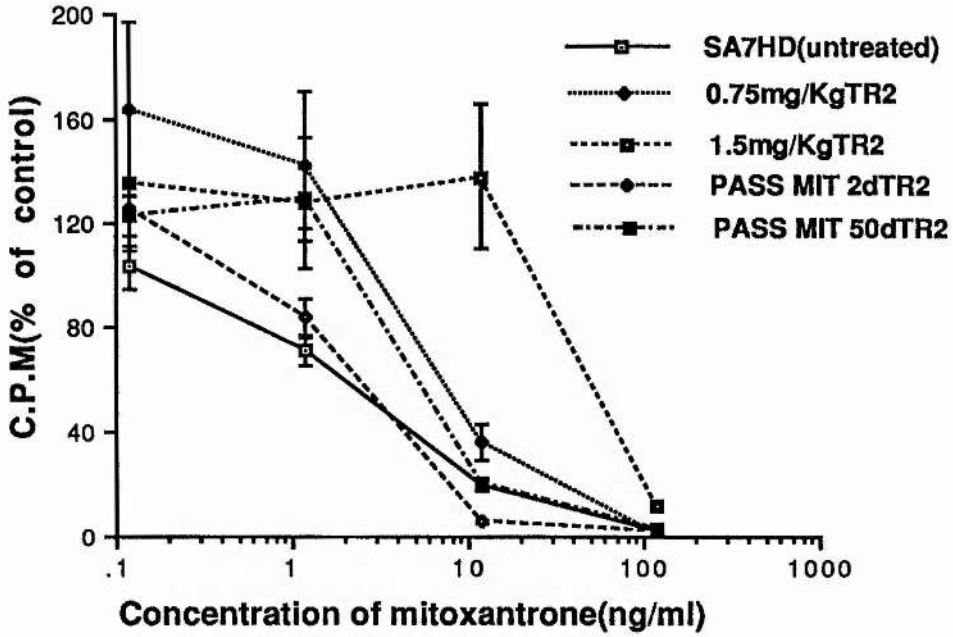


Fig.138:A comparison of mitoxantrone sensitivities of the various rec. leuks. Cytotoxicity was determined using (3H)-thymidine uptake assay in vitro.

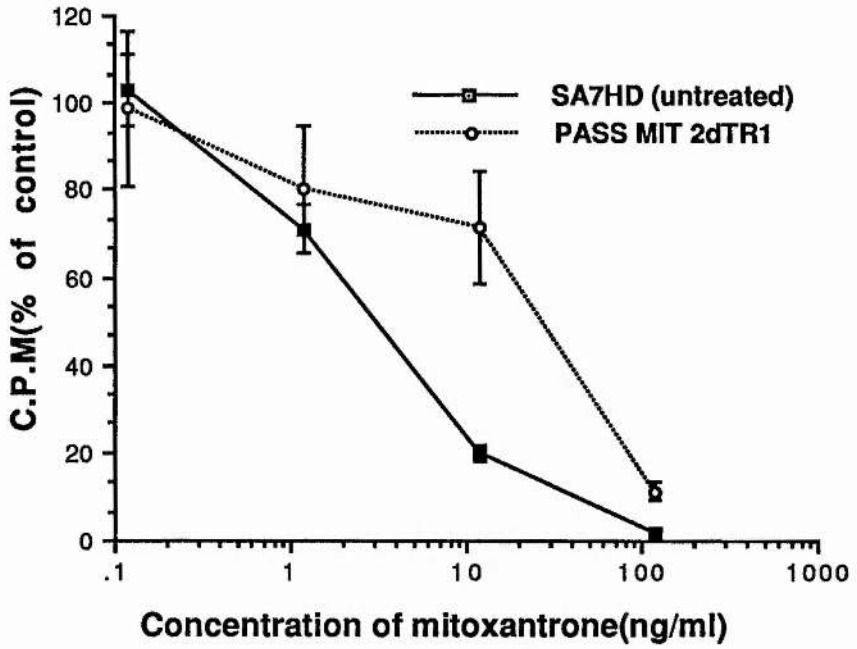


Fig.139: The response of rec. leukaemia(PASS MIT 2dTR1) to mitox. Cytotoxicity was determined using (3H)-TdR uptake assay in vitro.

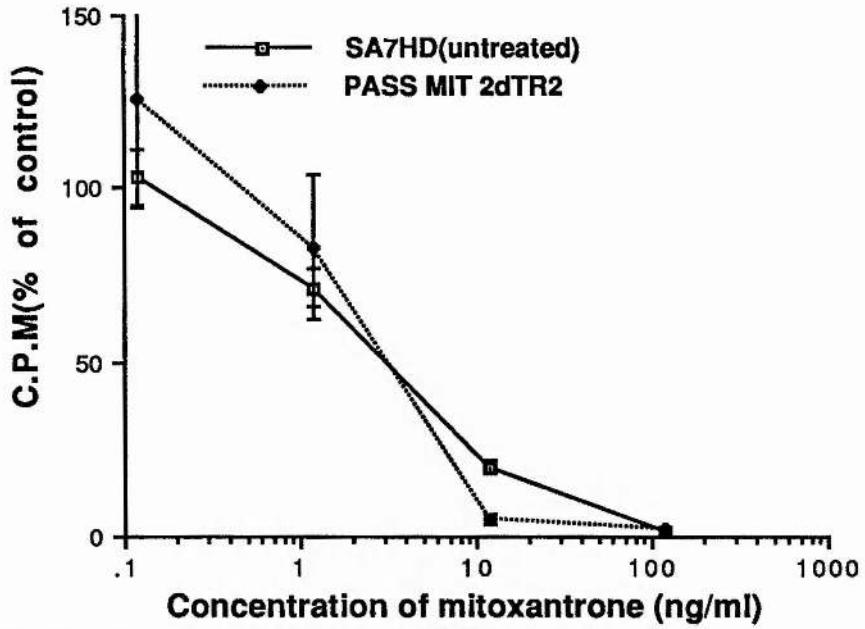


Fig.140: The response of PASS MIT 2dTR2 to mitoxantrone.
The rec. leukaemia was passaged twice
Cytotoxicity was determined using (3H)-thymidine uptake assay.

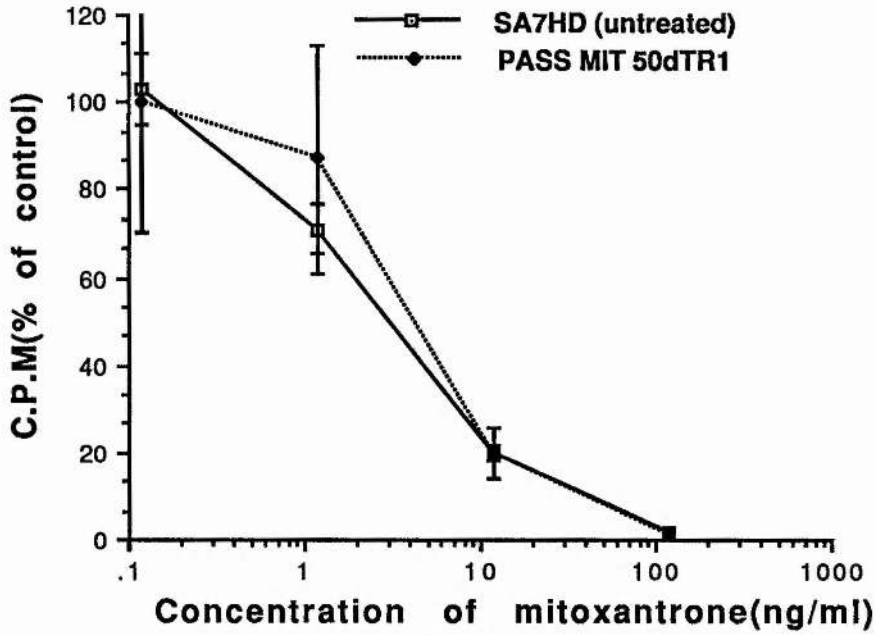


Fig.141: The response of PASS MIT 50dTR1 to mitoxantrone in vitro.
Cytotoxicity was determined using (3H)-thymidine uptake assay.

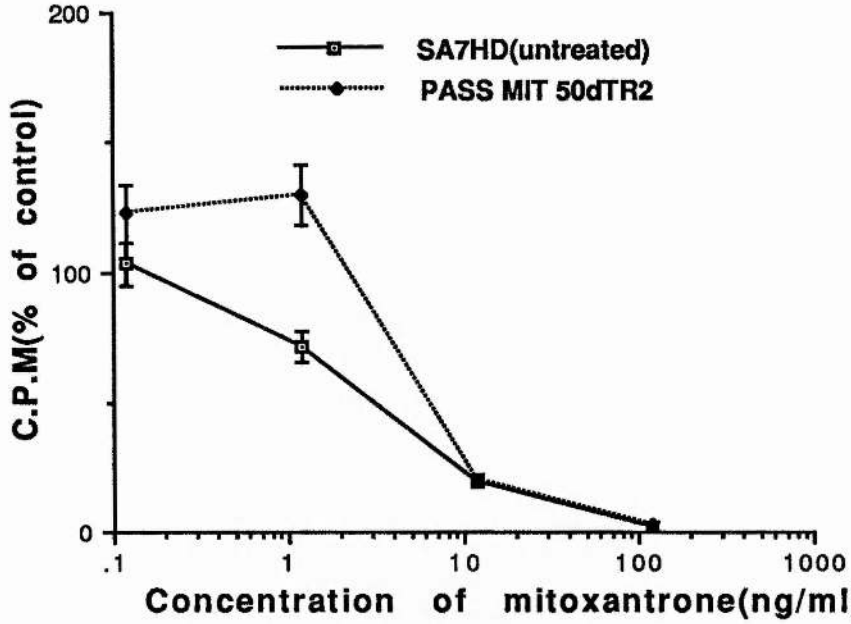


Fig.142: The response of PASS MIT 50dTR2 to mitoxantrone. The recurrent leukaemia was passaged twice. Cytotoxicity was determined using (3H)-TdR uptake assay in vitro.

5.2.24 Differential Count of Recurrent Leukaemic Bone Marrow Cells and Untreated Leukaemic Bone Marrow Cells. Tables 10 and 11 show (respectively) the differential count of untreated and recurrent leukaemic bone marrow cells following treatment of SA7HD with 0.4mg/kg mitoxantrone. Both leukaemias had nearly identical generalised blast count (23.68 vs 28.94%) and percentage of myeloid cells. Thus, recurrent leukaemic cells were not morphologically different from untreated leukaemic cells (Plate 10). (Compare with Plate 7).

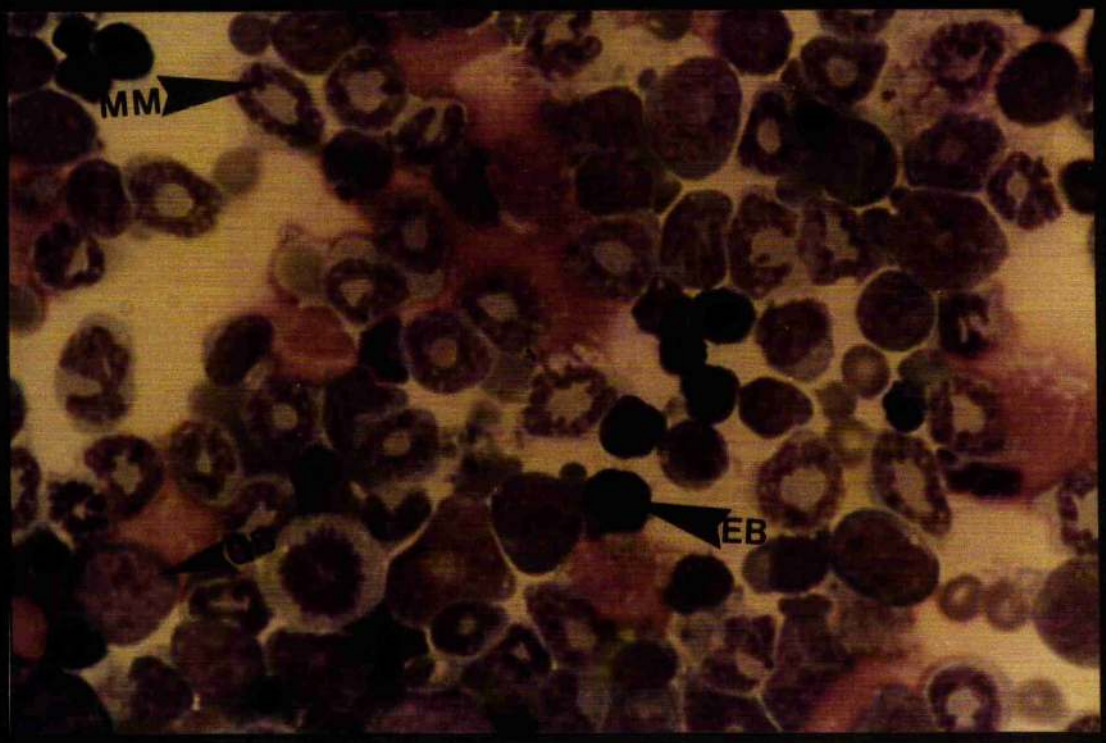


Plate 9: Bone marrow cells of a normal CBA/H mouse. Very few immature cells are evident. EB= erythroblast; GB = generalised blast; MM= metamyelocyte (magnification x 1300).

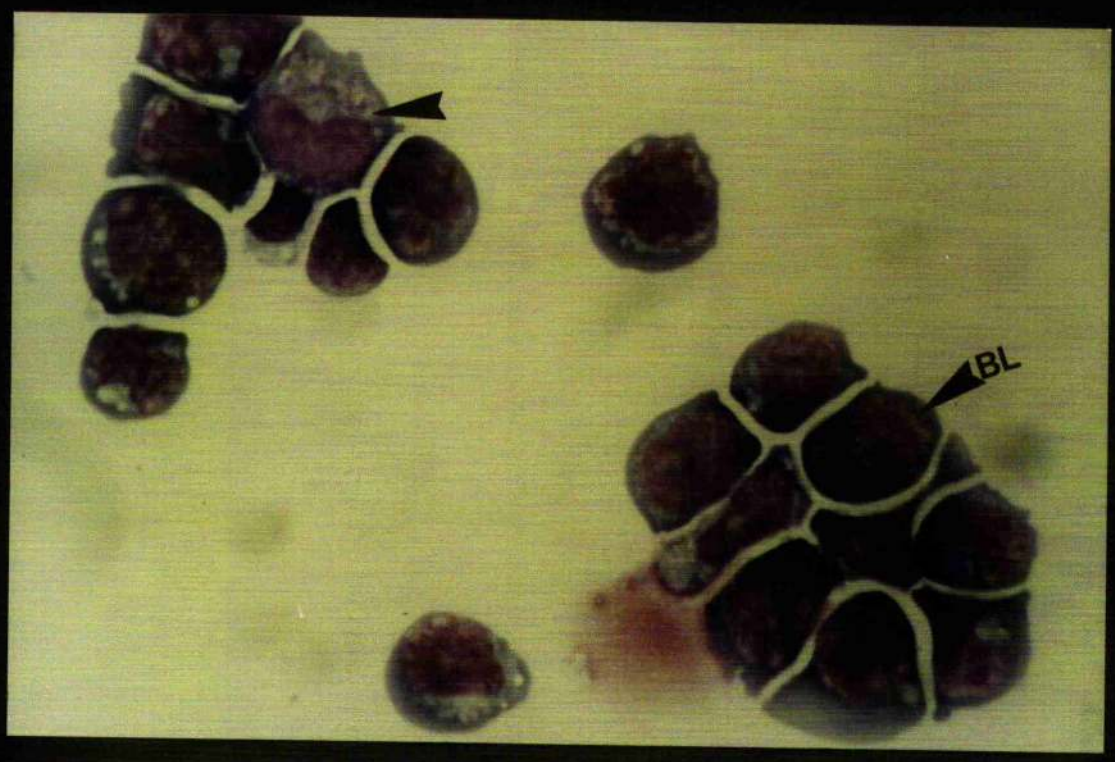


Plate 10: Leukaemic bone marrow cells of a recurrent leukaemia following treatment of SA7HD with 2 doses of mitoxantrone (0.4mg/Kg). The differential count of these bone marrow cells is identical to that of untreated (SA7HD)leukaemic bone marrow. BL= blast cells. (Magnification x 1300).

Table 10 : Differential count of bone marrow cells from untreated SA7 high cell dose transplant cell line.

	% Total	*% Haem*
Generalised blasts	23.68	26.67
Myeloid cells	49.01	55.19
Monocytes	2.96	3.33
Erythroid cells	2.30	2.59
Lymphocytes	6.58	7.41
Damaged cells	0.99	1.11
Unclassified cells	0.99	1.11
Other cells	2.30	2.59
Tissue cells	11.18	

	% Myeloid
Myeloblasts	34.45
Promyelocytes	12.92
Myelocytes	21.05
Metamyelocytes	27.27
Band cells	3.83
Segmented cells	0.48

Table 11 : Differential counts of bone marrow cells from recurrent leukaemia following treatment of SA7HD cell line with 2 doses of mitoxantrone (0.4mg/Kg (I.P)).

	% Total	% Haem. cells
Generalised blasts	28.94	35.0
Myeloid cells	47.27[a]	57.0
Monocytes	1.29	1.0
Erythroid cells	0.00	0.0
Lymphocytes	1.29	1.1
Damaged cells	0.96	1.1
Unclassified cells	0.96	1.1
Other cells	1.93	2.3
Tissue cells	17.36	-
	% Myeloid cells from (a) above	
Myeloblasts	37.97	
Promyelocytes	29.11	
Myelocytes	27.43	
Metamyelocytes	5.49	
Band cells	0.00	
Segmented cells	0.00	

5.3 The Effect of Mitoxantrone on Normal Mice:

The aim of the following investigation is to investigate the toxic effect of mitoxantrone on the haematopoietic system of normal mice. In addition, bone marrow cells of in vivo treated normal mice were monitored for their response to treatment with mitoxantrone in vitro.

5.3.1 Experimental procedure: Normal CBA/H female mice weighing 20-25g were injected (I.P) with the following doses of mitoxantrone on days 1 and 5: 0.4, 0.75, 1.5, 2 and 3mg/kg. Groups of 5 mice per dose were used. The following haematological parameters were determined on days 4, 8, 16, 30 and 60 following the last mitoxantrone injection: haematocrit(PCV), femur cellularity, spleen weight, haemoglobin(Hb) and number of GM-CFC's per 5×10^4 treated bone marrow cells. In addition, the response of the bone marrow cells to subsequent mitoxantrone treatment in vitro was investigated, using the tritiated thymidine uptake assay (section 3.2). Similarly, the response of normal bone marrow myeloid progenitors of the (in vivo) treated mice to mitoxantrone treatment in vitro was determined using GM-CFC assay.

Results

5.3.2 Effect of Mitoxantrone on Haematopoietic System of Normal Mice:

Mitoxantrone in the doses studied had no marked effects on spleen weight, Hb, PCV, femur cellularity and myeloid progenitor number in normal mice (Tables 12 a and b). Even with the highest dose used (3mg/kg), the most noticeable effect was a depression of spleen weight to about 60% of untreated values (Table 12a). However, none of the mice died from any drug-related toxicity in an observation period of nine days.

This contrasts sharply with the toxicity observed with the same dose of mitoxantrone (3mg/kg) in leukaemia bearing mice whereby all the mice died from drug toxicity.

Table 12a : The effect of mitoxantrone on spleen weight, haemoglobin and haematocrit of normal CBA/H mice.

Mice received two doses of mitoxantrone on days 1 and 5.

	Spleen Weight (mg)				*Haemoglobin (g/L)*				*P C V (%)*			
Days	*4*	*8*	*16*	*60*	*4*	*8*	*16*	*60*	*4*	*8*	*16*	*60*
Dose(mg/kg)												
Control	65±6	63±1	69±7[a]	-[b]	15.0	15±0.2	14±0.5	-	45±1	43±0.5	44±1	-
0.4	65±3	67±2	63±0.4	-	14 ±0	15±0.3	14±04	-	43±1	43±0.4	43±0.	-
0.75	-	-	-	-	-	-	-	14.2	-	-	-	39
1.5	59+3	52± 0	57+1.5	-	14±0	14± 0	14±0.1	-	43±1	40±0.5	40±0.3	-
3.0	-	39±1	-	-	-	15±0	-	-	-	39±1.0	-	-

a. Mean and standard error of 5 determinations.

b. (-) not determined.

Table 12b : The effect of mitoxantrone on femur cellularity and myeloid progenitor cell numbers in normal CBA/H mice. Mice received two doses of mitoxantrone on days 1 and 5.

	Femur cellularity ($x10^6$)				*GM-CFC*			
Days	*4*	*8*	*16*	*60*	*4*	*8*	*16*	*60*
Dose (mg/kg)								
Control	14.0	13	12	-	39±2[a]	45±3	45±2	(-)[b]
0.4	9.0	13	13	-	65±4	65±3	49±3	-
0.75	-	-	-	13	-	-	-	40
1.5	9.0	10	13	-	51±2	44±2	49±3	-
3	-	12	-	-	-	-	-	-

a. Mean ± SE of five determinations.

b. (-) = not determined.

5.3.3 Response of In Vivo treated Bone Marrow Cells to Mitoxantrone Treatment In Vitro: Bone marrow cells of normal mice that received mitoxantrone (0.75mg/Kg) in vivo 48 hours previously, developed protective effect towards subsequent mitoxantrone exposure in vitro (Figure 143). However, no such protective effect was developed by bone marrow cells of leukaemic mice that received mitoxantrone (0.75mg/Kg) 24 hours before the mice became moribund with leukaemia (Figure 143). The $I.C_{50}$ of the treated normal bone marrow cells was 60ng/ml (Figure 143) as compared to 6ng/ml for untreated NBM cells.

Figure 144 (mean of 3 experiments) shows a comparison of responses of bone marrow cells of mitoxantrone treated (0.4mg/kg) and untreated normal mice to Ara-C alone or in combination with mitoxantrone (1.2ng/ml) in vitro. Bone marrow cells from treated mice were less sensitive to Ara-C alone as compared to untreated NBM cells. Similarly, they were markedly less sensitive to the combination of Ara-C and mitoxantrone. Similar insensitivity to mitoxantrone alone or in combination with Ara-C was observed (Figure 145)(mean of 3 experiments).

The decreased sensitivity to mitoxantrone persists for 30 days after mitoxantrone administration. The responses of bone marrow cells to Ara-C alone or in combination with mitoxantrone from mice treated 30 days previously with 2 doses of mitoxantrone (0.75mg/Kg) are shown in Figure 146. Response to Ara-C alone was unchanged, however, when mitoxantrone (1.2ng/ml) was added, the cells became markedly less sensitive to the combination as compared to untreated cells (Figure 146).

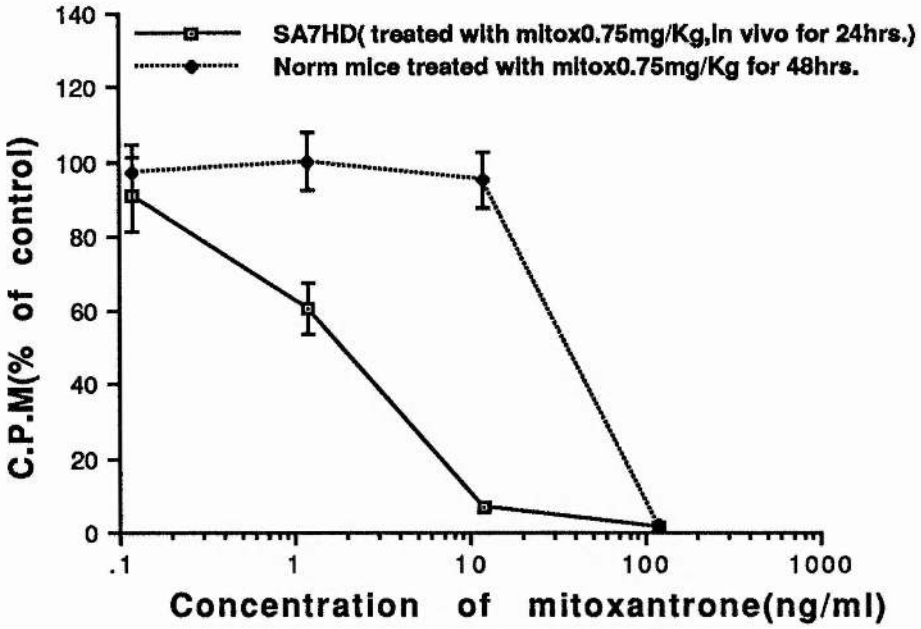


Fig. 143: A comparison of in vitro mitoxantrone sensitivity of in vivo pretreated normal and leukaemic bone marrow cells. Cytotoxicity was determined using (3H)-TdR uptake assay in vitro.

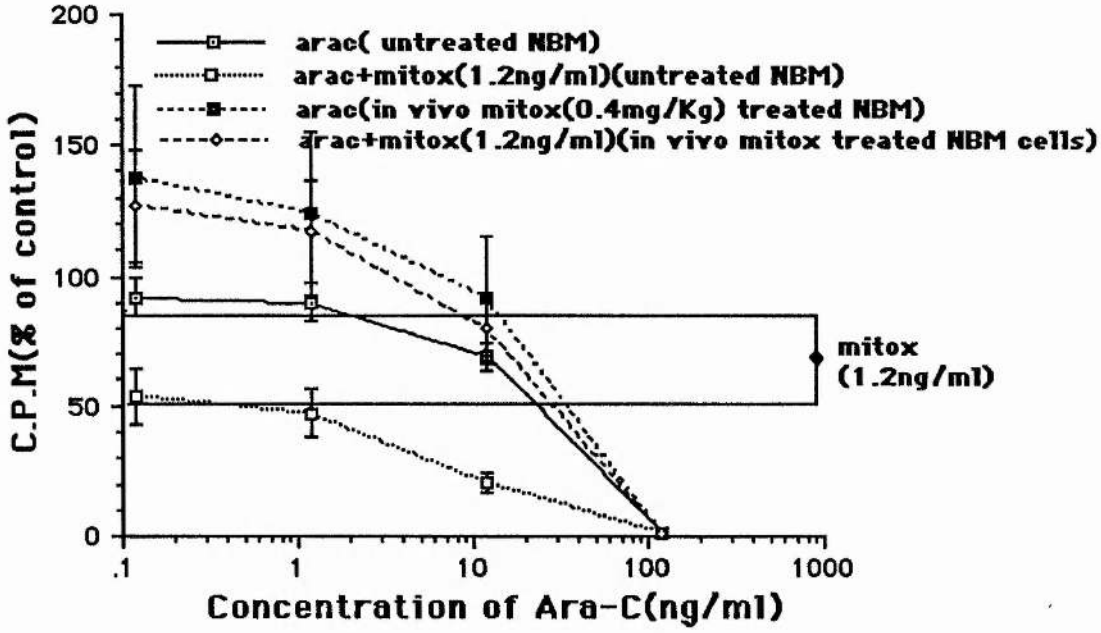


Fig.144: A comparison of invitro mitoxantrone sensitivity of in vivo mitox(0.4mg/kg) pretreated and untreated NBMcells. Cytotoxicity was determined using the (3H)-thymidine uptake assay.

The treated cells were also less sensitive to mitoxantrone alone and in combination with Ara-C (1.2ng/ml) (Figure 147). The $I.C_{50}$ of mitoxantrone, against treated cells was still unchanged at 60ng/ml, while that of untreated cells was 6ng/ml (Figure 147).

When mice were treated with a higher dose of mitoxantrone (2mg/kg), the bone marrow cells of such mice were as sensitive as bone marrow cells from untreated mice to Ara-C alone or in combination with mitoxantrone (1.2ng/ml) (Figure 148). Similarly, sensitivity to mitoxantrone alone or in combination with Ara-C (1.2ng/ml) was unchanged in the treated bone marrow cells (Figure 149). Similar responses as described for 2 mg/Kg were observed in bone marrow cells of mice treated with 3mg/Kg mitoxantrone. Thus, no change in sensitivity was observed in response to Ara-C with or without mitoxantrone (Figure 150) and mitoxantrone with or without Ara-C (Figure 151). In addition, there was no difference in IC_{50} of mitoxantrone against treated and untreated normal bone marrow cells.

The protective effect developed by bone marrow cells following treatment of normal mice with mitoxantrone (0.75mg/Kg) lasts up to 60 days after the last mitoxantrone injection.

Figure 152 shows the response of such bone marrow cells to Ara-C alone or in combination with mitoxantrone (1.2ng/ml) indicating that they were less sensitive as compared to untreated(NBM) cells. Similarly, Figure 153 shows response of the treated cells to mitoxantrone as compared to untreated cells. Again the IC_{50} of mitoxantrone against treated cells was 60ng/ml white IC_{50} against untreated cells was 6ng/ml. Thus, the protective effect begins from as early as 48 hours following administration of mitoxantrone (0.75mg/Kg) and lasts for as long as 60 days.

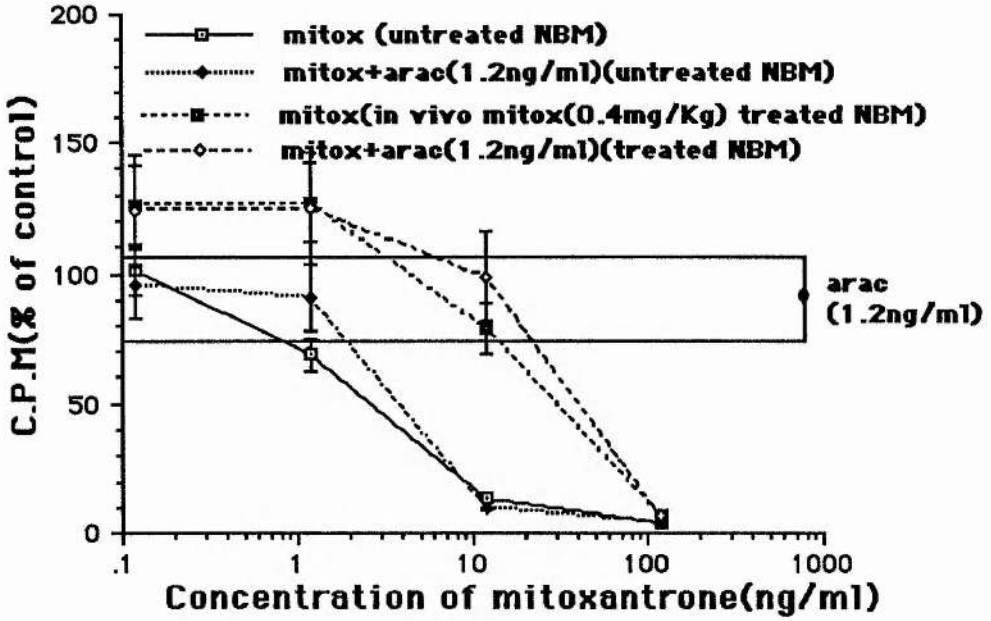


Fig.145: The response of in vivo treated and untreated NBM cells to in vitro treatment with mitox alone or in combination with arac. Cytotoxicity was determined using the (3H)-thymidine uptake assay.

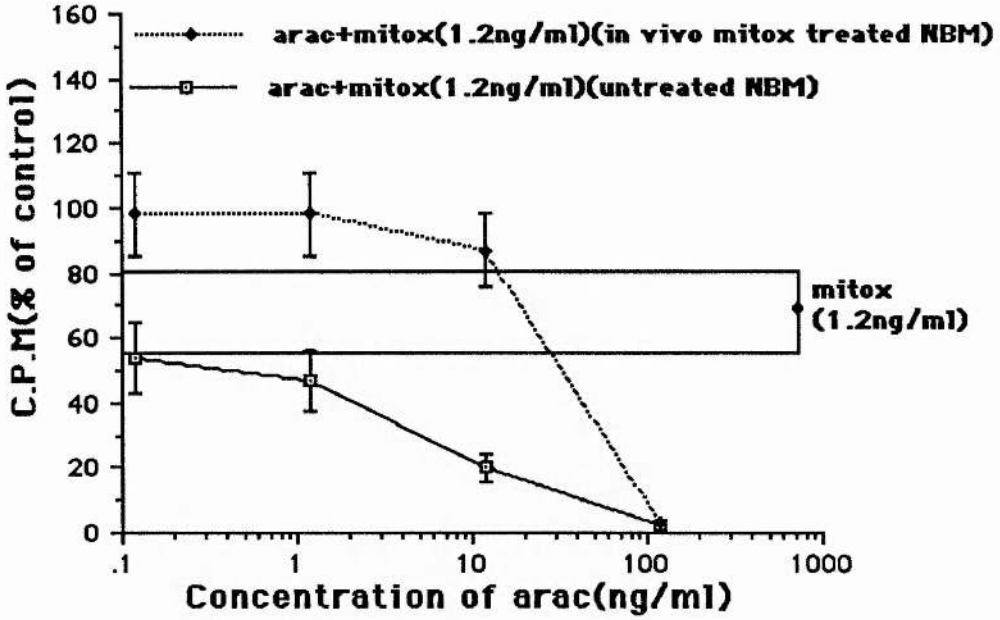


Fig.146: A comparison of the response of in vivo mitox treated and untreated NBM cells to in vitro treatment with combination of arac and mitoxantrone. The pretreated mice received 2 doses of mitox(0.75mg/Kg) 30 days previously.

5.3.4 Response of Myeloid Progenitors from In Vivo Treated Bone Marrow Cells to Mitoxantrone Treatment In Vitro: Bone marrow cells of mice treated with mitoxantrone (0.4, 0.75 and 2mg/Kg) in vivo were tested for the response of myeloid progenitors to subsequent

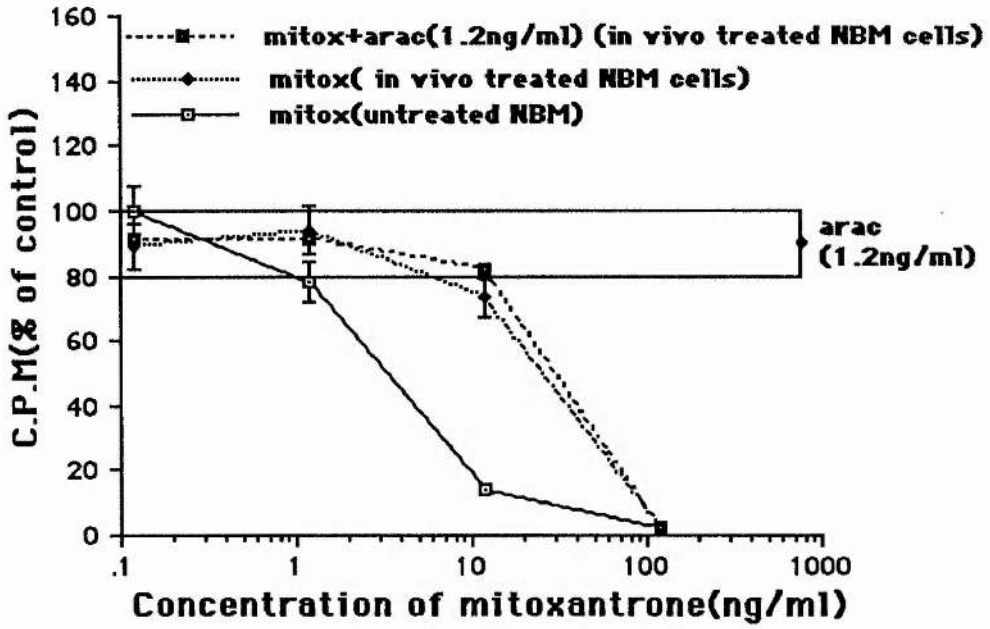


Fig.147: The response of in vivo treated and untreated NBM cells to in vitro treatment with mitox alone or in combination with arac. The pretreated mice received 2 doses of mitox(0.75mg/Kg) 30 days previously.

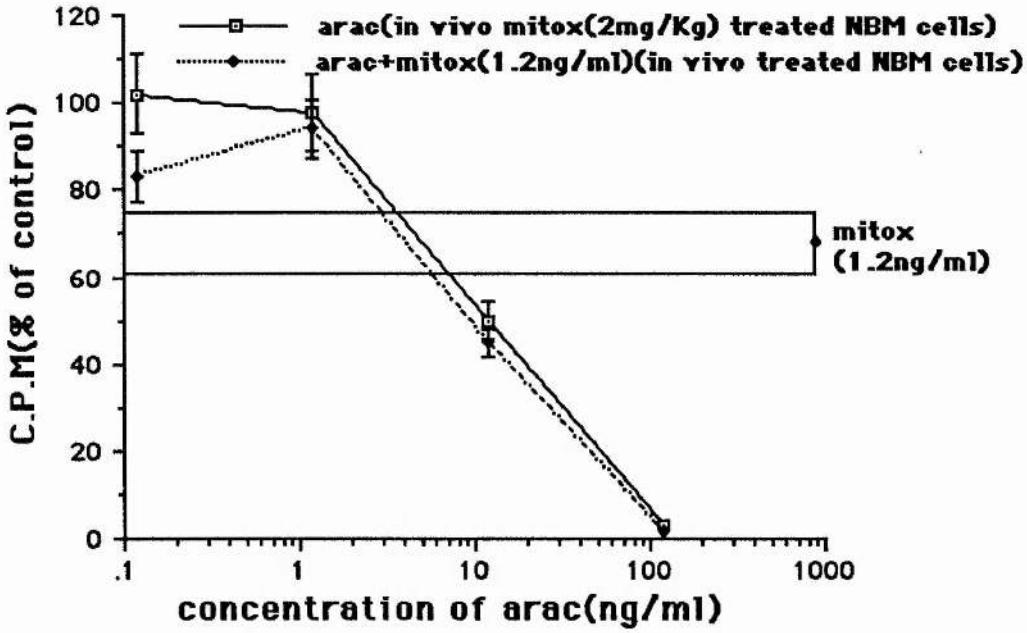


Fig.148:The response of in vivo mitox(2mg/Kg) treated NBM cells to in vitro treatment with arac alone or in combination with mitox. Cytotoxicity was monitored using the (3H)-thymidine uptake assay.

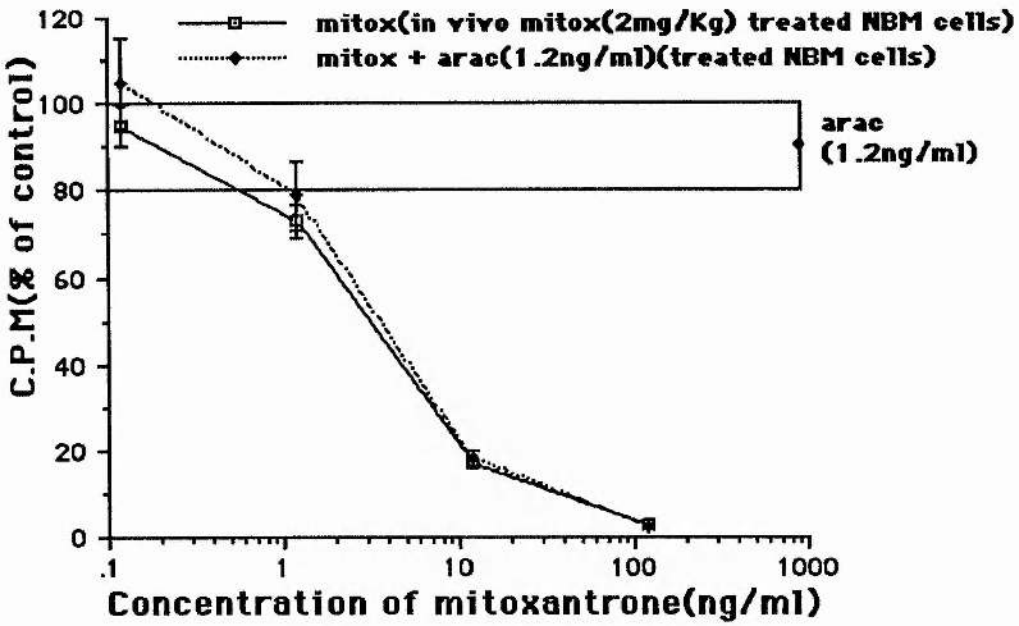


Fig.149: The response of in vivo mitox (2mg/Kg) treated NBM cells to in vitro treatment with mitox alone or in combination with arac. Cytotoxicity was determined using the (3H)-thymidine uptake assay.

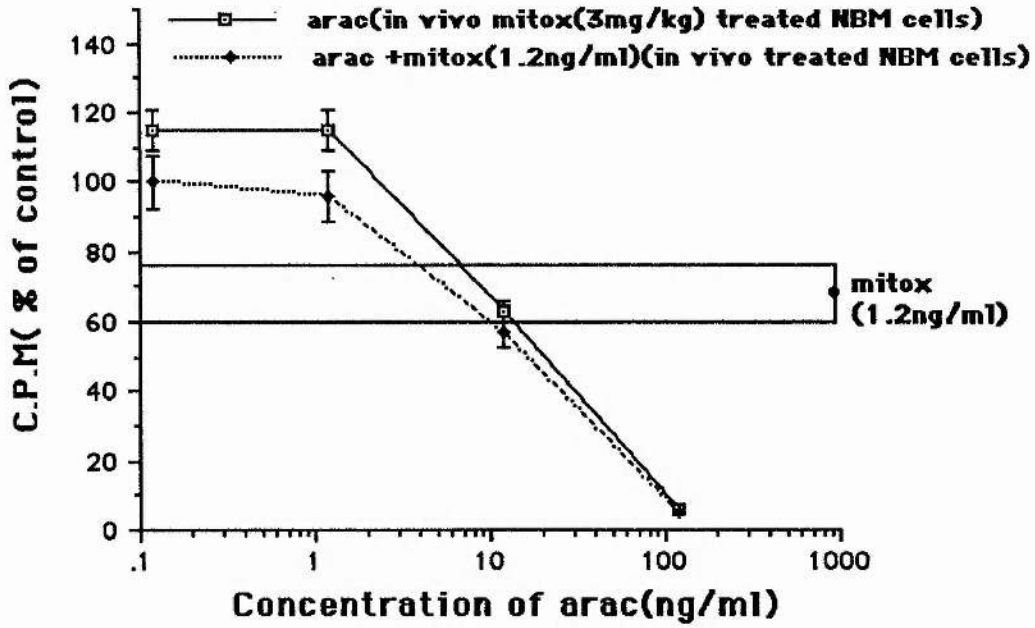


Fig.150:The response of in vivo mitox(3mg/Kg) treated NBM cells to in vitro treatment with arac alone or in combination with mitox. Cytotoxicity was determined using the (3H)-thymidine uptake assay.

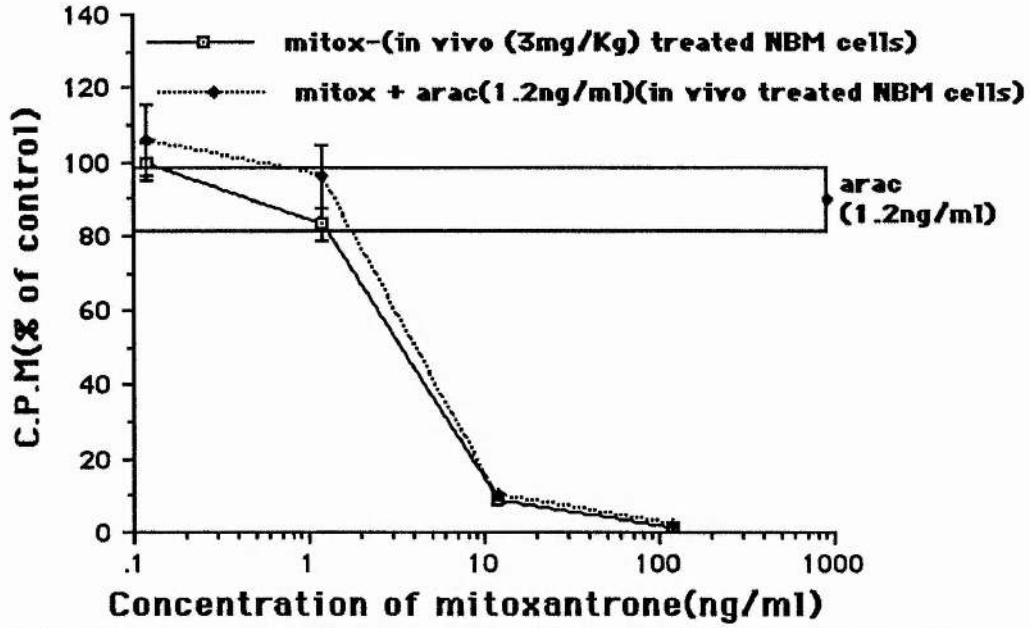


Fig.151: The response of in vivo mitox(3mg/Kg) treated NBM cells to in vitro treatment with mitox alone or in combination with arac. Cytotoxicity was determined using the (3H)-thymidine uptake assay.

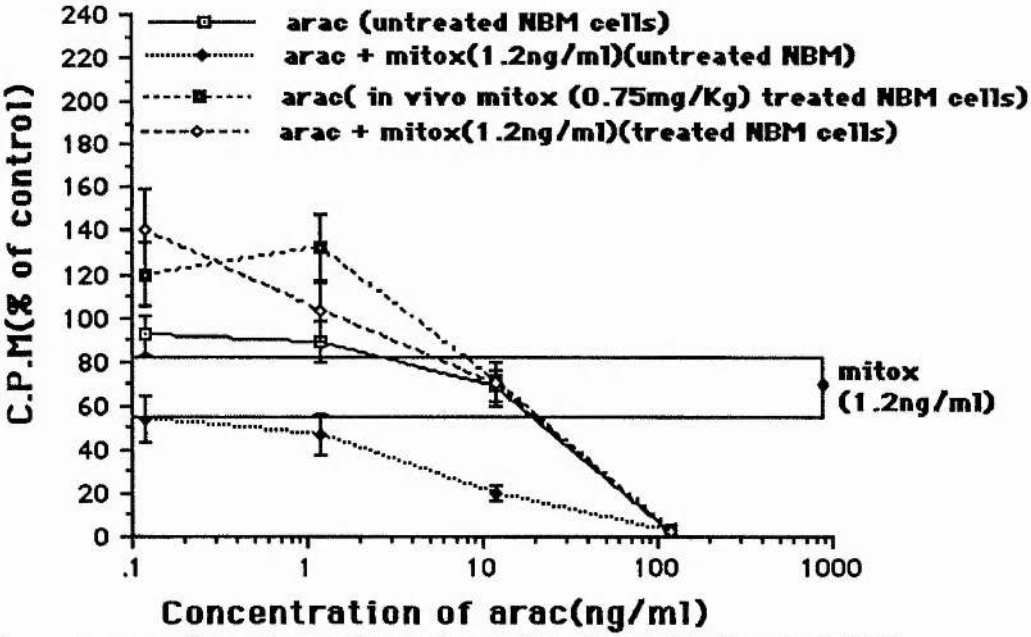


Fig.152: The response of in vivo mitoxantrone treated and untreated NBM cells to in vitro treatment with arac alone or in combination with mitoxantrone. Norm mice were pretreated with 2 doses of mitox(0.75mg/Kg) 60 days previously.

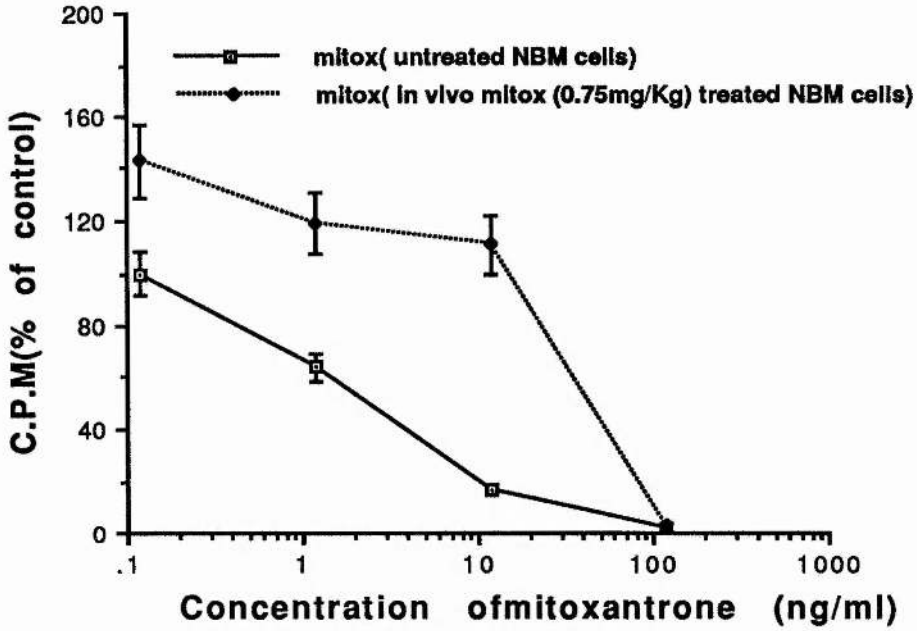


Fig.153: A comparison of the response of in vivo mitoxantrone treated and untreated NBM cells to in vitro treatment with mitoxantrone. Norm mice received 2 doses of mitox(0.75mg/Kg) 60 days previously.

mitoxantrone treatment in vitro. Figure 154 shows that in mice treated with mitoxantrone(0.4mg/Kg or 0.75mg/Kg), myeloid progenitors apparently exhibit decreased sensitivity to subsequent mitoxantrone treatment in vitro . In bone marrow cells of mice that received two doses of mitoxantrone(0.75mg/Kg) , the protective effect was evident even after 60 days following the last mitoxantrone injection.

It is noteworthy that bone marrow cells of mice cured of leukaemia by mitoxantrone treatment also exhibit protective effect towards subsequent mitoxantrone exposure in vitro. In addition, doses of mitoxantrone (2-3mg/kg) that resulted in loss of protective effect by bone marrow cells of treated normal mice were also toxic to leukaemia bearing mice (Table 1). Moreover, bone marrow cells of a mouse dying in remission from drug related toxicity lacked protective effect towards mitoxantrone exposure in vitro. However, although 2mg/Kg administered to normal mice resulted in loss of protective effect, bone marrow cells of mice cured of

leukaemia with identical dose of mitoxantrone (2mg/Kg) (Table 1) still developed protective effect against subsequent mitoxantrone exposure in vitro (Figure 86).

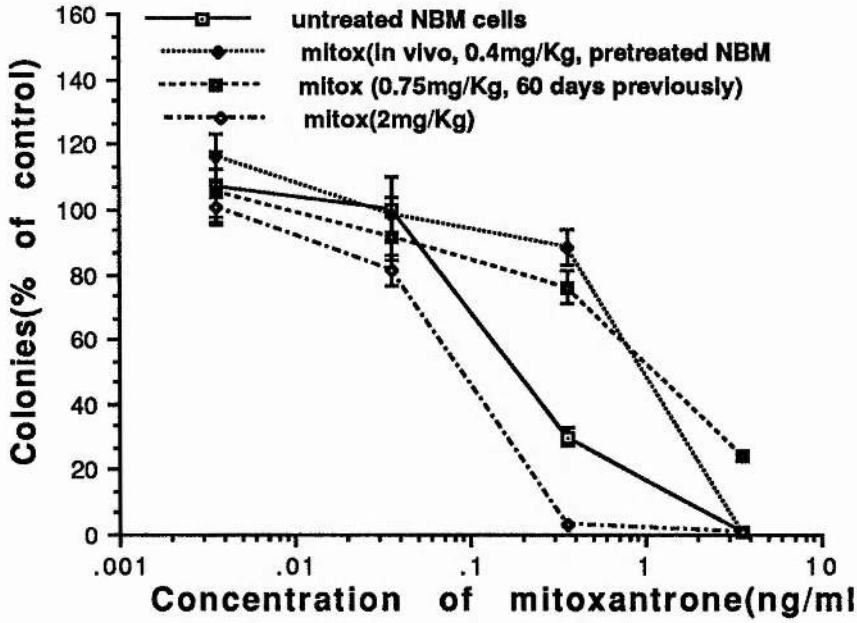


Fig. 154:The resp of myeloid progenitors of invivo treated and untreated NBM cells to subsequent treatment with mitoxantrone in vitro. Norm mice were pretreated with 0.4, 0.75, or,2 mg/Kg mitoxantrone.

6. DISCUSSION

CHAPTER SIX

6. DISCUSSION

6.1 Choice of In Vitro Drug Concentrations and Mode of Drug Exposure

The choice of drug concentrations for use in in vitro assays is a difficult one. However, generally either one-tenth the peak plasma concentration or the concentration x time (C x T) is used. The C x T of an anticancer drug is one of the most important pharmacokinetic parameter in determining drug efficacy and toxicity (Alberts, Chen and Salmon, 1980). The peak plasma concentration of Ara-C following I.V administration of 100mg/m^2 in humans was 10μg/ml and the C x T was 340 - 430ng hr/ml (Alberts, Chen and Salmon, 1980). Similarly, Spiro et al 1981 have determined that 38ng/ml Ara-C would inhibit 90% of human myeloid colony forming cells in vitro. The C x T for mitoxantrone was 36ng hr/ml (Alberts et al, 1985) and one-tenth peak plasma range concentration lies in the range of 5 - 10ng/ml. The concentrations of the two drugs used in this study (0.12 - 120ng/ml) lie between one-tenth and peak plasma concentration and C x T values reported above.

The next decision following determination of the drug concentrations was the mode of drug exposure. Again, it is difficult to determine what mode of drug exposure - one hour pulse or continuous incubation - mimics the clinical situation. One hour pulse exposure can lead to falsely high resistance rates for cycle specific drugs. However, it has been stated that one hour treatment approximates serum peak levels after bolus administration (Drewinko et al, 1981). On the other hand, continuous drug exposure may yield falsely high sensitivity rates. In one study, up to 92% correlation with in vivo result was obtained when

continuous incubation was employed as opposed to 71% correlation with one hour pulse (Park et al, 1983). Both types of drug exposure have been employed in this study.

After deciding drug concentrations, mode of drug exposure, it was necessary to determine next, the type of assay to be employed in assessing drug-induced cell kill. As no single assay is perfect it is better to use different methods that provide complementary information rather than a single method (Rosenblaum et al 1980). In this study both clonogenic and nonclonogenic assays have been employed. It has been suggested that because the dose-limiting toxicity of cytotoxic drugs is toxicity to normal tissues, it is necessary to compare drug effects on both leukaemic and normal bone marrow cells (Park et al, 1983). Therefore, effects of both cytosine arabinoside (Ara-C) and mitoxantrone (mitox) on leukaemic cell lines and normal (murine) bone marrow cells were determined. In clinical practice, combinations of drugs rather than any single drug are used in the treatment of leukaemia. In this study, the effect of combinations of Ara-C with mitoxantrone or mitoxantrone with Ara-C were monitored. However, it has been emphasized that it was not important whether a particular drug combination was additive, synergistic or subadditive. What was important was whether there was a differential effect between tumour and normal tissue (Brown and Rasey, 1980).

Most in vitro drug effects on tumour cells are investigated using cell culture techniques. The latter provide a rapid, and economical method of determining the effects of antitumour agents. However, a major drawback is extrapolating in vitro effects to the situation in vivo. Whereas cells in culture usually grow exponentially, a large fraction of tumour cells in vivo are in quiescent (G_O) state. The latter are usually considered less sensitive than proliferating cells to the lethal activity of

most antitumour drugs (Drewinko et al, 1981). The difference in cell killing efficacy between proliferating and stationary cells is generally attributed to the ability of quiescent cells to repair drug-induced damage before it became a fixed lethal event at mitosis (Ikebuchi et al, 1988).

6.2 Effect of Serum on Drug Activity In Vitro

Serum is usually added to media for growing cells in vitro. In this study, foetal calf serum was employed. It has been reported that the interaction of antitumour drugs and serum albumin could have a major influence on drug pharmacology and efficiency (Takahashi et al, 1980). Cytotoxic agents were divided into three categories depending on effect of serum on their activity : (a) compounds such as adriamycin and mitomycin C whose biological activities were decreased in the presence of serum albumin; (b) those that were unaffected such as Ara-C, actinomycin D, fluorouracil and (c) those whose biological effects were increased in the presence of serum eg bleomycin, vincristine and vinblastine (Takahashi et al, 1980). In this study, serum did not appear to affect mitoxantrone cytotoxicity in vitro (Figure 9).

6.3 Differential Staining Cytotoxicity (DiSC) Assay

Staining of cells with vital dyes has been traditionally used as an indicator of cell death following cell membrane damage (Roper and Drewinko, 1976). However, care must be exercised since the dye could be excluded by both living and reproductively dead cells .

The timing suggested by Weisenthal et al, (1983 b) (4 days) was adopted (in this study) in order to allow for drug-induced damaged cells to become membrane permeable while at the same time minimising loss of control viability.

One problem of the cell viability (DiSC) assay is that cells that survive drug treatment may continue to proliferate during the time it takes lethally damaged cells to lose membrane integrity. Thus, by the time dead cells exclude the dye, the surviving cells may have proliferated to such an extent that the percent cell kill is grossly underestimated. In addition, some lethally damaged cells may undergo early disintegration so that they will not be available to stain with the dye at the end of the assay. The use of an internal standard (of Pigeon red cells in this study) has overcome the problem of proliferation of surviving cells and / or disintegration of damaged cells in culture. An elegant example from Weisenthal et al, (1983b) serves to illustrate the significance of including an internal standard of duck red blood cells (DRBC's).

The criteria for an in vitro response in the dye exclusion assay was defined as reduction of the viable tumour cell number to 30% or less of the control value (Weisenthal et al, 1983b). Similarly, a cut off point of 30% tumour cell viability (TCV) was used by Bosanquet et al, (1983) in in vitro - in vivo correlations. Using these criteria, none of the leukaemic cell lines studied (SA2, SA7 and SA8) was sensitive to Ara-C below 120ng/ml (Figures 2, 4 and 6). Even when mitoxantrone (1.2ng/ml) was added, no increased effect was observed. Similarly, the SA2 line was sensitive only to the highest concentration of mitoxantrone used (120ng/ml) (Figure 3), while the SA7 and SA8 (Figures 5 and 7 respectively) seem more sensitive at lower concentrations. It has been suggested that TCV below 25% would predict for partial or complete response in vivo (Bosanquet et al, 1983).

Although there was qualitative agreement between cell viability assay and cell number assessment using electronic particle counter in this study, there were quantitative differences particularly with the SA2 cell line (Figures 2 and 3). This may be because the electronic particle count

probably included incompletely lysed dead cells which stain with fast green (Weisenthal et al, 1983 b).

Some of the drawbacks of the dye exclusion method include the fact that it is difficult to distinguish between tumour and nontumour cells particularly in bone marrow preparations. Also, the assay may be incapable of detecting cell growth inhibition which is not followed by cell death (Weisenthal et al, 1983). This is a potential problem with cell cycle specific agents. The vital dye assay based upon the ability of "viable" cells to exclude trypan blue was found in one report to indicate cytotoxic effect of treatment for only one of four drugs tested. Adriamycin, cisplatin and vincristine elicited an insignificant reduction (<5%) in the proportion of viable cells, even though these drugs reduced survival by more than one log when measured by a clonogenic assay (Rupniak and Hill, 1980). However, Weisenthal et al, (1983 b) have cautioned against labelling the results of either assay as "correct" or "incorrect" and care is needed in comparisons of two different assays. It is possible that the more sensitive assay may overestimate cell kill or else the less sensitive assay may underestimate cell kill. Rather than a direct comparison of two assays using the same drug concentrations, each assay may require different range of drug concentrations.

The advantages of a dye exclusion assay include simplicity, and the fact that it can be employed to assess drug induced cytotoxicity in quiescent cells (Bosanquet et al, 1983). In comparison to cloning assays which only work with 25% of specimens, the assay can be used to determine drug effect in in vitro nongrowing neoplasms such as chronic lymphocytic leukaemia and the assay has found wide application in other tumour systems as well. There are correlations reported with results of both clonogenic assay and clinical response (Bosanquet et al,1983; Durkin, Ghanta and Hiramoto, 1983). In addition, because results are available

in a shorter time (as compared to colony assays), it may be used to monitor patient's response in the clinic.

6.4 Tritiated Thymidine Uptake Assay

The rate of nucleic acid synthesis has been used as a measure of the proliferative activity of a tumour. Therefore, the ^{3}HTdR uptake assay can be employed to determine the proliferation dependent cytotoxic effect of antitumour drugs (Volm, Maas and Mattern, 1980). It is assumed that the amount of radioactive precursor (eg ^{3}HTdR) incorporated by the cells is proportional to the extent of DNA synthesis. And zero incorporation of ^{3}HTdR means zero viability (Freshney, Paul and Kane, 1975). It has been suggested that exposure to drug in this assay should cover at least two cell cycle times. And conditions must be such that control cultures maintain logarithmic growth throughout the assay. Finally, sufficient time must be allowed for delayed cytotoxicity to be expressed (Freshney, Paul and Kane, 1975). In this study, leukaemic bone marrow cells or normal bone marrow cells were pulsed with [3H]TdR, four days following either continuous incubation or 1 hour pulse with drugs.

The ability of an in vitro assay to predict in vivo activity depends on the criteria used to define in vitro sensitivity. In one study, the cut-off level of drug sensitivity was arbitrarily defined as the concentration inhibiting 50% of [^{14}C]-thymidine uptake ($I.C_{50}$). It was claimed that this level roughly corresponded with 50% colony inhibition with many different types of cytotoxic agents. Therefore, if the $I.C_{50}$ of the test drug is lower than the pharmacologically achievable plasma concentration, the drug is considered as being active (Nakayama et al, 1989). Applying this criteria to the present results, both Ara-C and mitoxantrone would be expected to be active in vivo since they inhibited [3H]TdR uptake in the

leukaemic cell lines in vitro using concentrations that were below peak plasma concentrations of the drugs in humans. However, in order for the assay to predict for clinical responses in humans, a stricter criteria was suggested. Greater than 80% suppression of ^{3}HTdR uptake was employed as a criteria in one study (Maddox et al, 1984).

A common finding in this study was that very low concentrations of both Ara-C and mitoxantrone stimulated radioactive precursor uptake (to above control levels) rather than inhibited ^{3}HTdR uptake following both 1 hour pulse or continuous incubation with either of the two drugs (Figures 15, 20 and 39-43 respectively). This was also observed by other workers who found that very low concentrations of daunorubicin and 6-thioguanine stimulated precursor uptake in some blast cells using both thymidine and leucine uptake assays. (Sanfillipo et al, 1981). At low concentrations (10^{-10} - 10^{-9}M) adriamycin causes stimulation of growth. Early work (Wang et al, 1972) demonstrated that adriamycin stimulated [^{3}H]-thymidine incorporation into DNA at low doses. Similarly, Roper and Drewinko, 1976 also noted over 50% stimulation of DNA synthesis by low concentrations of adriamycin. In all these cases, higher concentrations resulted in cytotoxicity. Although adriamycin causes up regulation of receptors for a growth-stimulating substance (Zuckier and Tritton, 1983) this effect occurs only at concentrations which cause cytotoxicity and thus will probably not explain growth stimulation by the drug (Vichi and Tritton, 1989). It has been suggested that cytotoxicity is the opposing counterpart to growth stimulation and common cellular mechanisms may govern both processes (Vichi and Tritton, 1989).

The limitations of the ^{3}HTdR uptake assay include the following facts: different batches of animal sera contain different amounts of endogenous thymidine which will compete with [3H]TdR for

incorporation into newly synthesized DNA. Many suppliers of serum do not provide an estimate of the concentration of thymidine in the serum (Larcom and Smith, 1988). Also ^{3}HTdR could be converted to thymine during the incubation period. The cells being examined in short term culture may not be representative of the whole in vivo cell population(Maddox, 1984). Non-tumour cells for example, stromal cells that are also proliferating may incorporate radioactive label (Twentyman, Walls and Wright, 1984).

Changes in [3H]TdR uptake may be either due to a change in the number of cells taking up the label or the amount of thymidine incorporated by each cell as the two cannot be distinguished using scintillation counting. In addition, there may be drug-induced alteration in nucleoside transport. Thymidine transport through cell membrane seem susceptible to attack by agents capable of alkylating sulfhydryl groups involved in transport reaction (Maurer, 1981). Non-dividing cells that are undergoing DNA repair could incorporate exogenously added [3H]TdR and this will obviously not correlate with cell division. Furthermore, the re-utilisation of ^{3}HTdR following catabolism of DNA from dead cells is a significant process in rat bone marrow (Maurer, 1981). It has been reported that exogenously administered [3H]TdR is incorporated exclusively via the salvage pathway (Maurer, 1981). However, the salvage pathway of DNA synthesis is important for proliferation of tumour cells (Hirose et al, 1987). The salvage pathway is important for the supply of nucleosides for DNA synthesis in some tumour cells since nucleosides reverse the toxic effects of some drugs e.g methotrexate (Hirose et al, 1987).

The main advantages of the [3H]-thymidine uptake assay include simplicity, speed and it is less tedious than comparable assays such as autoradiography. The [3H]-thymidine uptake assay has been employed

to determine the presence of acquired drug resistance in tumours. Leukaemic cells from patients with resistant disease showed significantly decreased sensitivity to Ara-C, daunorubicin and 6-thioguanine alone or in combination when response was monitored using [3H]TdR uptake assay (Viano et al, 1986). It has been suggested that if colony size was reduced by drug treatment, it may not be noticeable in a clonogenic assay, whereas in an isotope uptake assay it would be expected to result in reduced ^{3}HTdR uptake (Twentyman, Walls and Wright, 1984).

Similarly, a ^{3}HTdR uptake assay correlated with GM-CFC assay in mouse bone marrow cells (Horak, 1983). An increase in ^{3}HTdR incorporation reflected an increase in both progenitor numbers and their proliferative status. However, Twentyman, Walls and Wright, (1984) have warned that it should not be expected that two assays will necessarily produce identical dose-response curves.

In this study, the [^{3}H]-thymidine uptake assay was employed to determine the responses of leukaemic cell lines (81287, 10.12, 10.13, SA2, SA7 and SA8) and normal bone marrow (NBM) cells to continuous incubation with Ara-C, mitoxantrone alone or in combination.

6.4.1 Continuous Incubation with Ara-C

Increasing the concentration of Ara-C caused increased inhibition of [^{3}H]-thymidine uptake in all leukaemic cell lines and NBM cells using the continuous incubation method. This is in agreement with a report by Heinemann et al, (1988) showing that inhibition of DNA synthesis was linearly related to the intracellular concentrations of Ara-C. Even very low concentrations 10ng/ml kills NBM cells with prolonged exposure (Greenberg, Vernkersen and Mosny, 1976). At very low Ara-C concentrations the rate of Ara-C accumulation within cells was

dependent on rate of transport of the molecule across cell membrane. However, at high concentration the phosphorylation capacity of the cells determines the net uptake (White, Wrathmel and Capizzi, 1987).

The responses of 81287 leukaemia to both Ara-C and mitoxantrone changed with transplantation. Similarly, spleen cells appeared to be more sensitive to these drugs as compared to bone marrow cells. This may be as a result of different subpopulations of leukaemic cells residing in the two haematopoietic tissues. Whether the decreased sensitivity to Ara-C with transplantation of the 81287 cell line was due to a change in number of cells in S- phase or simply due to variation between experiments can only be speculated. It has been reported that sensitivity to Ara-C is not simply dependent on the number of cells in S-phase. This is because it must first be taken up by cells, phosphorylated and incorporated into DNA before any cell killing effect will be manifested (Rustum and Preisler, 1979). In addition, the cytotoxic effect of Ara-C must be interpreted with caution. This is because where isotopically labelled thymidine is used to follow DNA synthesis in the presence of Ara-C, erroneous values may arise due to competition between Ara-C and thymidine for transport (Mulder and Harrap, 1975). Despite this drawback, however, Ara-C sensitivity of cells from patients with leukaemia correlated with clinical outcome (Preisler et al, 1984).

The most noteworthy finding regarding continuous incubation with Ara-C in this study, it its lack of differential cytotoxicity. Ara-C was not more toxic to the leukaemic cell lines as compared to normal bone marrow cells (compare Figures 30, 32 and 34 with Figure 36). If anything, normal bone marrow cells seemed slightly more sensitive as compared to leukaemic cell lines (SA2, SA7 and SA8). This is supported by a study by Singer and Lynch, (1987). The beneficial effect of Ara-C in the clinic may be due to its ability to inhibit self renewal of leukaemic stem cells

(McCulloch et al, 1988). And the fact that the differential effect of chemotherapy is as a result of differences in recovery between normal and leukaemic cells (Singer and Lynch, 1987).

6.4.2 Continuous Incubation with Mitoxantrone

Leukaemic bone marrow cells from the cell lines 81287, 10.12, 10.13, SA2, SA7, SA8 and normal bone marrow cells were treated with mitoxantrone and the cytotoxicity produced determined using [3H]TdR uptake assay. As was observed with Ara-C, the responses of 81287 cell line changed with transplantation. Sensitivity apparently decreased with passaging although this was not marked with mitoxantrone. In addition, spleen cells were slightly more sensitive than bone marrow cells. Of the high cell dose transplant leukaemias (SA2, SA7 and SA8), the SA2 cell line seem most sensitive to mitoxantrone, followed by SA8 and lastly the SA7 cell line. No obvious explanation for this difference in sensitivity can be offered. As mitoxantrone enters cells by passive diffusion, it is unlikely that a difference in drug uptake can account for the differences in sensitivity (Haugstad, et al, 1987). The cytotoxic effect of mitoxantrone does not depend on the cell type or whether the cells are in proliferative or stationary phase, although it is apparently more effective during late 'S' Phase (Poirier, 1986). It binds DNA by intercalation and the degree of DNA strand breaks correlates with cytotoxicity (Alberts et al, 1985). In all leukaemic cell lines and normal bone marrow cells studied, mitoxantrone was more potent than Ara-C in vitro using comparable doses. However, Hill (1987), has cautioned against such comparisons without taking into account the doses of the drugs used clinically and also the possibility of metabolism or inactivation which may occur in vitro. Except on the SA2 cell line,

mitoxantrone did not exhibit selective toxicity on leukaemic cell lines as compared to normal bone marrow cells in this study. This is in agreement with the study reported by Schozel, Van Putten and Lowenberg, (1986), as well as Buick; et al, (1979) and Fountzilas et al; (1986). In one of the leukaemic cell lines (SA7), there was no marked difference between one hour pulse with mitoxantrone and continuous incubation with mitoxantrone. This is in agreement with the report that there was little or no dependence of cytotoxicity on duration of drug exposure (Johnson et al , 1983).

From these results, it would appear that mitoxantrone may be more potent than Ara-C in vivo. However, the efficacy of each drug will depend on the overall balance between antileukaemic effect and toxicity particularly against normal bone marrow cells.

6.4.3 Drug Combinations and One Hour Pulse Treatments

When mitoxantrone (1.2ng/ml) was added to Ara-C (0.12-120ng/ml), there was additive inhibition of [3H]TdR uptake in the vast majority of cell lines (81287T6, SA7, SA2, SA8, NBM), slight antagonistic effects in a few cell lines (SA7FT5, SA8FT14) and in only one instance was synergism observed (81287T10, Figure 20). In the latter case, the sensitivity of the leukaemia was changing with transplantation and therefore this observation may or may not be manifested with subsequent passaging. Similar additive to slightly antagonistic effects were observed when Ara-C (1.2ng/ml) was added to mitoxantrone (0.12-120ng/ml). It is possible to suggest explanation for the absence of synergy or consistent additivity with these drug combinations. If a drug alone completely inhibits DNA synthesis, there is no possibility of a synergistic effect by a second drug. Both mitoxantrone and Ara-C have the same molecular target : inhibition of DNA synthesis via

intercalation and inhibition of incorporation of nucleotides respectively. Viano et al,(1986) have suggested similar mechanism to explain the effect of combinations of daunorubicin, Ara-C and 6-thioguanine (6-TG). The most prominent observation regarding one hour pulse with Ara-C followed by addition of mitoxantrone either immediately or 24, 48 or 72 hours later was that SA7 and NBM were subject to the effects of drug scheduling, while SA2 and SA8 were unaffected. Synergistic effects were observed with SA7 and NBM cells when mitoxantrone was added 24 hours rather than immediately following one hour pulse with Ara-C. This may be because the cytotoxicity of Ara-C is highly schedule dependent (Heinemann et al; 1988). For example, two 3-hour exposure of HL-60 cells to Ara-C separated by a drug-free interval were more cytotoxic than continuous 6-hour exposure (Powell et al, 1988). Similarly, Kallman et al, (1989) have suggested that the effects of a second drug may depend on the cell cycle status of the cells surviving the first treatment. In addition, it was reported that killing of cells in S-phase by Ara-C may be followed by recruitment and syncronisation of the surviving cells (Colly, Peters and Willemze, 1986).

The effects of mitoxantrone and Ara-C were evaluated in vitro using the HL-60 cell line in one study. The cells were exposed for one hour to each drug either simultaneously or sequentially (up to 28 hours interval), washed free of the drug and seeded in replicate plates. Colony growth was observed on day 7. Simultaneous exposure of the two drugs produced additive cell kill. Ara-C followed by mitoxantrone produced time-dependent synergism. Up to 99% increase in cell kill was observed when mitoxantrone was added 8 hours or longer following Ara-C. Reversal of the order of exposure i.e mitoxantrone followed by Ara-C did not produce time-dependent synergism (Fountzilas et al, 1983). Similarly, in this study, pulsing NBM and SA7HD leukaemic cells with

Ara-C for one hour followed by addition of mitoxantrone after 24hrs was more cytotoxic as compared to when mitoxantrone was added immediately following a one hour pulse with Ara-C. A similar schedule-dependent effect was found to follow combinations of vincristine and methotrexate. However, it must be borne in mind that clinically active drug combinations do not necessarily produce additivity or synergism in vitro nor in vivo. Conversely, synergistic combinations in vitro may not always be therapeutically more effective (Kano et al; 1988).

6.5 Autoradiography

The labelling indices of the leukaemic cell lines SA2, SA7 and SA8 were determined both in the presence and absence of mitoxantrone. In all cases, there was a qualitative correlation between labelling index inhibition monitored using autoradiography and [3H]TdR uptake inhibition measured using scintillation counting. A similar correlation was reported by Lucke et al, (1986). However, at low concentrations of mitoxantrone in this study, there was no close correlation between inhibition of labelling index and [3H]TdR uptake suppression determined using scintillation counting. Similarly, Roper and Drewinko, (1976) reported that the labelling index method either overestimates or underestimates the degree of cell kill produced by adriamycin and BCNU respectively. And the labelling index method was found to consistently underestimate cell killing by methotrexate (Rupniak and Hill, 1980). In addition, labelling index determination is a tedious procedure. Moreover, a misleadingly high labelling index maybe obtained as cells killed by a drug undergo degeneration leaving only undamaged cells to be recognised and enumerated (Weisenthal, 1981). However, unlike scintillation counting, the labelling index

method gives information on which cells are labelled in a tissue composed of different cell types (Aherne, Camplejohn and Wright, 1977). In addition, the labelling index does provide a guide to the proliferative activity of a cell population.

6.6 Granulocyte-Macrophage Colony Forming Cell (GM-CFC) Assay

The responses of normal bone marrow myeloid progenitor cells to one hour pulse and continuous incubation treatments with Ara-C and mitoxantrone were monitored in vitro. In all doses and schedules studied, mitoxantrone was much more toxic to normal bone marrow cells as compared to Ara-C. One hour pulse with mitoxantrone produced virtually the same effect as continuous incubation with Ara-C using comparable doses in vitro. In support of this observation is the report that mitoxantrone causes greater myelosuppression than even doxorubicin (Okunewick, Buffo and Kociban, 1985). In vitro drug-induced inhibition of GM-CFC has been reported to correlate with in vivo myelosuppression (Hug et al; 1984). It has been established that bone marrow represents the dose limiting tissue for most cytotoxic agents. It has been stated that only agents that affect tumour cells more profoundly than bone marrow cells can effectively reduce tumour burden (Hug et al, 1984). It has been reported that the cytotoxic effects of Ara-C on mouse and human GM-CFC's were in general similar (Greenberg, Vankersen and Mosney, 1976). Ara-C was not specifically more toxic to acute myeloid leukaemia colony forming units (AML-CFU) as compared to GM-CFC (Greenberg, Vankersen and Mosny, 1976; Singer and Lynch, 1987) . However, Ara-C was reported to exhibit increased specificity for chronic-myeloid leukaemia colony forming units (CML-CFU-C) as compared to GM-CFC. The heightened sensitivity

of CML-CFU-C to Ara-C was likely to be as a result of either differences in drug uptake or phosphorylation but not due to number of cells in S-phase. The lack of differential sensitivity to hydroxyurea, a phase-specific agent supports this claim (Spiro et al, 1981). The beneficial effect of Ara-C in AML may be due to the ability of the drug to suppress not only terminal division but also self-renewal of leukaemic stem cells. Self renewal inhibition by Ara-C is determined using suspension culture, and correlates better with clinical outcome as compared to inhibition of terminal division in methylcellulose (McCulloch, 1988).

Mitoxantrone like Ara-C is myelosuppressive in humans. It also does not display differential toxicity towards AML-CFU as compared to GM-CFC (Schozel, Van Putten and Lowenberg, 1986; Buick et al, 1987; Fountzilas et al, 1986).

Hug et al, 1984, have suggested that the GM-CFC assay could be employed to determine appropriate drug concentrations for use in new drug screening against tumour cell lines in vitro. Thus, in vitro tests meant to predict clinical response should employ dose ranges based rationally on bone marrow tolerance. In this way false positive predictions from testing too high concentrations and false negative predictions from testing at very low concentrations may be avoided (Hill, 1987).

A major problem with the colony assay is that normal cell to cell contact may be minimal. And colony growth was found to show marked variation even between incubators. In addition, there could be significant loss of water from the system during incubation. When a colony assay was employed to measure the sensitivity of tumours to drugs, clinical correlations have been obtained, making many authors suggest that it should be the standard with which other assays are to be compared (Salmon 1983; Mattox and Von Hoff, 1980; Chang, 1983; Selby,

Buick and Tannock, 1983; Von Hoff, 1983A). However, the caution advised by Weisenthal and Lippman (1985), must be borne in mind. These include poor reproducibility of test results between different laboratories and subjectivity of colony counts. In addition, there is a possibility that tumour stem cells may be nondividing in vivo while cells forming colonies are necessarily dividing cells. Lastly, clonogenic assays measure cell kill over a narrow log range while meaningful clinical responses require multiple-log cell kill (Weisenthal and Lippman, 1985).

6.7 Responses of Leukaemic Cell Lines to Growth Factors Monitored In Vitro

WEHi myelomonocytic leukaemia conditioned medium and L-cell conditioned medium were used singly and in combination to stimulate the in vitro proliferation of leukaemic cell lines in this study. The WEHi-3 cell line synthesizes multi- colony stimulating factor (Interleukin-3, IL-3) (Metcalf, 1985). Two distinct forms of IL-3 have been purified from WEHi-3-conditioned medium. The two have identical amino acid sequence but one is shorter by six amino acids at the N-terminal. The relation between the two is not fully understood although the full length form may be more active (Murthy et al, 1989). The L929 fibroblast cell line is a crude source of macrophage colony stimulating factor (CSF-I; M-CSF) (Pragnell et al, 1988). When murine bone marrow cells were incubated with ^{125}I-CSF-1 for 6 hours, 50-70% of myeloblasts, promyelocytes and small numbers of late granulocytic cells were labelled (Shadduck et al, 1983). The primary leukaemic cell line 81287 (Figures 70 and 71) and low cell dose transplant of SA7 cell line (SA7FT5, Figures 74 and 75) were not very responsive to WEHi, L929 or their combinations in vitro. In contrast, the high cell dose transplant

SA8 cell line was relatively more responsive to single and combinations of WEHi and L929 conditioned media (Figure 76). This difference in sensitivity between low cell dose and primary leukaemia on one hand and high cell dose passage leukaemia on the other, may be due to the relatively closer similarity in morphology between low cell dose transplant and primary cell lines (Hepburn et al, 1987). As was observed in this study, acute myeloid leukaemias respond to growth factors by an increase in proliferation. Because of the intrinsic abnormalities in leukaemias, CSF-stimulated proliferation results in self- generative divisions rather than divisions leading to the production of differentiating progeny (Metcalf, 1985). This increase in proliferation is usually manifested by an increase in DNA synthesis (Salem et al, 1989). A dose-response relationship exists between CSF concentration and magnitude of proliferation (Metcalf, 1985).

Responses of Leukaemic Cell Lines to Combinations of Growth Factors

Despite their lineage specificities, colony stimulating factors (CSFS) do produce synergistic effects when they are combined (Falk and Vogel, 1988). For example, in both normal and 5-fluorouracil treated murine bone marrow cells, recombinant granulocyte-macrophage colony stimulating factor (rGM-CSF) synergises with CSF-I to produce large colonies following 10-12 days incubation (Falk and Vogel, 1988). This is as a result of the stimulation of murine macrophage progenitors with a high-proliferative potential (HPP-CFC) (Bradley and Hodgson, 1979). A similar synergistic effect was observed following combinations of IL-3 with CSF-I both in vitro and in vivo (Williams et al, 1987). Even in serum-free medium, combinations of multi-CSF and M-CSF increased the size of colonies. The number of granulocyte-macrophage progenitor cells responding to the combination is greater than the number

responding to the individual growth factors (Mortsyn and Burgess, 1988). The commonest methods for studying the synergistic effects of cytokines include colony formation in agar as well as proliferative responses measured by [3H]TdR incorporation (Falk and Vogel, 1988). A similar synergistic effect was displayed by SA2 leukaemic cell line with combinations of WEHi and L929 conditioned media in both serum containing and serum free media (Figures 72 and 78 respectively).

Growth factors have also been used to increase the cytotoxic effects of Ara-C. Ara-C exposure inhibits the proliferation of a higher proportion of AML clonogenic cells in cultures pretreated with growth factors than in controls (that were not growth factor treated) (Lista et al, 1989). The increased proliferative effect as a result of combinations of WEHi and L929 could explain the slightly enhanced effect observed in this study with Ara-C and mitoxantrone against SA7 leukaemic cell line in vitro (Figures 134 and 135).

The ^{3}HTdR incorporation assay may be more sensitive for measuring CSF responses than methylcellulose colony cultures, since activation of DNA synthesis was more frequently observed than induction of colony formation (Delwel et al, 1988). A comparison of the results of ^{3}HTdR incorporation and colony cultures showed that growth factor responses were less frequently evident in the methylcellulose colony assay. In five cases reported, significant amount of [3H]TdR was incorporated whereas no colonies formed. These differences were attributed to the fact that ^{3}HTdR uptake was measured on day-4 (similar to this study), whereas with the colony assay only a minority of cells that give rise to progeny following several cell divisions during 12-14 days of incubation is assayed. In addition, there is evidence to support the idea that AML cell growth may be better maintained in liquid culture systems than in semi-solid cultures. Moreover, in the cell suspension cultures, the cells settle

at the bottom of the microtitre plate thus enhancing cell to cell contact which may enhance AML growth (Delwel et al, 1988). The microtitre (^{3}HTdR uptake) assay is also faster and easier to evaluate than conventional colony counting (Horak et al, 1983).

6.8 Effect of Serum and Transferrin on Proliferative Response of SA2 Leukaemic Cell Line

The SA2 leukaemic cell line was capable of proliferating in vitro without the requirement for an exogenous supply of growth factors (Figure 77). The autocrine secretion of growth factor(s) has been postulated to result in the continuous proliferation of certain malignant cells (Weiss, 1983). It is generally accepted that serum plays an important role in ensuring the growth of most cell types in culture. Serum is a complex mixture and many serum components are poorly characterized. Moreover, there is usually a marked variation in some serum components between batches (Barnes and Sato, 1980). Although it may not be applicable to all cell types, certain cells reportedly grow better in media supplemented with horse serum as compared to foetal calf serum (Yoshimara et al, 1989). An example is THP-1 human monocytic leukaemia cell line. In the absence of serum, the SA2 leukaemic cell line was unable to proliferate in vitro. This suggests that substances present in the serum could be providing the leukaemia with growth stimulus. For example, a growth promoting factor in horse serum was identified as horse serum transferrin (Yoshimara et al, 1989). Similarly, cell lines which were hormone dependent in vivo did not show hormone dependent growth in vitro unless serum added to the culture medium was first depleted of hormones. The explanation was

that serum provided a mixture of hormones which was stimulatory for cell growth. Thus, hormones present in serum were masking effects of any supplementary hormones (Barnes and Sato, 1980). A similar effect could be operating with the SA2 leukaemic cell line as serum may contain colony stimulating factors. This partly explains the use of serum-free media by some investigators (Delwel et al, 1988).

The fact that transferrin did not provide the stimulus for the growth of the SA2 leukaemic cell line in vitro suggests that it is not the activity in serum that was responsible for the growth of the leukaemic cells. Transferrin seems to be essential for the proliferation of many cell types in vitro (Forsbeck and Nillsson, 1985). It has been shown to act as a growth factor for lymphocytes and small cell lung cancer cells (Forsbeck and Nillsson, 1985). The major function of iron binding proteins including transferrin is storage and transport of iron (Taetle and Honeysett, 1986). In addition, transferrin receptor is present on all dividing cells and is increased in cycling cells but absent in quiescent cells (Spooncer and Whetton, 1985).

It was suggested by some investigators that serum could be providing colony stimulating factors that were required for the growth of a megarkaryoblastic cell line in vitro (Yoshida et al, 1986). Inspite of the fact that in the presence of serum, the SA2 leukaemic cell line can grow in vitro with no exogenous growth factor added, synergistic proliferative effect was still observed following combinations of WEHi and L929. Similarly, two cases of erythroleukaemia and one case of myelomonocytic leukaemia grew in vitro without addition of growth factors. Yet, increased colony formation was observed when CSF was added (Yoshida et al, 1986). In addition, although colony formation by WEHi-3 cells in vitro was not dependent on stimulation by CSF, the

number and size of colonies were increased by the addition of CSF (Metcalf, Moore and Warner,1969).

6.9 Responses of Low Cell Dose Transplant and High Cell Dose Transplants of SA7 Cell Line to Mitoxantrone Treatment In Vivo

The SA7 is a myeloid leukaemia cell line and its morphological characteristic has been reported (Hepburn et al, 1987). The low cell dose transplant variant is slower growing than the high cell dose transplant (Tables 2(a) and 3(a)). During the terminal stages, both high cell dose transplant and low cell dose transplant leukaemic cell lines present with inhibition of normal haemopoiesis which is particularly severe with the low cell dose transplant (Tables 3 and 4). It has been reported that following injection of leukaemic cells into syngeneic mice, leukaemic cells proliferated in the bone marrow, spleen, liver and peripheral blood (Nara et al, 1984). Leukaemic cells may have "homing" receptors that function not only to provide adherence to bone marrow stroma but also support leukaemia cell survival and growth in the bone marrow environment . As the leukaemia develops in the bone marrow, the number of normal colony forming cells (GM-CFC's) and fibroblastoid colony forming units (CFU-F) decreased (Nara et al, 1984). The fact that the mice continued to live for a few days more when all the bone marrow cells were replaced by leukaemic cells may be due to persistent haematopoietic activity in the spleen and other extramedullary sites. Normal haemopoiesis is suppressed by physical displacement of stem cells by leukaemic cells and/or by production of inhibitory humoral factors such as acidic isoferritins (Broxmeyer et al, 1981).

Acute myeloid leukaemia (AML) and acute lymphocytic leukaemic (ALL) are clinically distinct diseases with different presentations and

prognosis. Yet in the preclinical screening of drugs for use in the treatment of AML, lymphoid murine models (P388 and L1210) are usually employed. In addition, the two leukaemias have different patterns of tissue infiltration and respond differently to chemotherapeutic agents. Even the rate at which the two leukaemias incorporate $^{35}S_{04}$ was found to be significantly different (Theodorakis and Goldberg, 1983). In this study, the response of SA7 myeloid leukaemia to mitoxantrone treatment in vivo was studied. This will then be compared to the responses of both P388 and L1210 leukaemias reported in the literature (Baumgart et al, 1988).

There was an increase in life-span and some mice were even cured with mitoxantrone in the dose range 0.75-2mg/Kg (IP-IP, Table 1). The maximum increase in life-span (%ILS) (including one long term survivor) of 146% was observed when mitoxantrone (1.5mg/Kg) was administered (I.P) using the SA7 high cell dose transplant. Similar response was obtained with identical dose of mitoxantrone (I.P) in the treatment of the low cell dose transplant of SA7 cell line (IV-IP, Table 3). In contrast, using same range of doses and schedule, a different response was observed with the P388 leukaemia . The optimal dose in P388 was 3mg/Kg which produced an average 175% increase in life-span and was curative to an average of 50% of the treated mice (Johnson et al , 1983). In this study, all tumour bearing mice that received 3 mg/Kg mitoxantrone died from drug toxicity, with no evidence of leukaemia. In addition, no increase in life-span over untreated control was observed in these mice (Table 1). When the dose of mitoxantrone in P388 leukaemia was reduced to 1.5mg/Kg, there was an average of 150% ILS with a 30% long term survivors. In a similar study but with the drug administered on days 1, 3 and 5, Fujimoto and Ogawa, 1982, observed that the optimal dose of mitoxantrone was 1.6mg/kg (IP-IP). With that

dose all five mice treated survived for 60 days. This suggests that this acute myeloid leukaemia model is less sensitive to the effect of mitoxantrone. The criteria for selecting candidate drug in the preclinical screening process has been reported by Venditti, 1981. The lower efficacy end point for preclinical screening (IP-IP) is T.C%> 120 and 125 (ILS > 20%) for P388 and L1210 leukaemias respectively. The higher efficacy T.C% is 175 for P388 and 150 for L1210. T is mean or median survival time of treated mice x 100 divided by the survival time of controls (C). The % increase in the life-span over control (% ILS) is equal to T.C%-100. Thus, applying this criteria for the SA7 cell line, mitoxantrone has reached the lower efficacy but not the higher efficacy end point. However, it is likely that the response of the SA7 cell line actually mirrors the clinical experience with mitoxantrone. For example, mitoxantrone when used singly was capable of inducing remission in only 20% of patients (Gale and Foon, 1986) and in one study, none of the patients receiving a single bolus infusion of mitoxantrone achieved complete remission (Prentice et al, 1984).

In the SA7 cell line there was no proportionality between dose and response. That is to say, doubling the dose within the therapeutic range was not accompanied by a proportional increase in life-span or cure of the treated mice. In a similar study using the rat brown Norway myeloid leukaemia model (BNML), Ara-C was shown to exhibit a weak dose-response relation. For example, a five fold increase in the dosage of Ara-C was only accompanied by a slight prolongation of the survival time (Rachel et al, 1988). It has been suggested that the relationship between cell kill and tumour response is extremely complex and varies from one drug to another (Twentyman, 1980). For example, in the B16 melanoma, 90% of the clonogenic cells could be killed by CCNU without causing a significant delay in tumour growth. In contrast, adriamycin

can produce a very considerable tumour growth delay without causing measurable degree of cell kill (Twentyman, 1980). Furthermore, when mitoxantrone was administered to SA7 cell line in vivo at 3mg/Kg body weight, all mice died as a result of drug toxicity. This suggests a very narrow therapeutic margin (0.75mg/kg to 2mg/kg) above which toxicity supersedes any antileukaemic activity and below which, no measurable antileukaemic effect was observed (Table 3). Indeed, steep dose-response curves are characteristic of many antitumour drugs. This severely restricts the degree of tumour cell kill that can be achieved by a course of chemotherapy (Griswold, 1986). In addition, cell-killing by many drugs follow first-order kinetics and therefore it is a constant fraction of and not absolute number of cells that is killed by a particular drug dose. Thus, a dose that would reduce 10^4 sensitive cells to 10^1 would also reduce 10^9 to 10^6 (Griswold, 1986). However, although no strict dose-response relationship exists there is still a relationship between the number of leukaemic cells inoculated and amount of drug necessary to obtain curative effect. The dose necessary to achieve complete response or cure increases with the number of clonogenic cells (Hug et al, 1984).

In this study, the route of administration of leukaemic cells (I.V or I.P) had a marked effect on therapeutic outcome. For example, decrease in life-span was observed when leukaemic cells were injected I.V with mitoxantrone administered I.P as compared to the IP-IP regime (compare Tables 1 and 2). Similar observation was made by Johnson et al, 1979. They reported that mitoxantrone was less effective when (P388 and L1210) leukaemic cells were injected I.V with mitoxantrone administered I.P. Moreover, as in this study, no long term survivors(cures) were seen with the I.V-IP regime. Similarly, whereas 4/6 mice bearing L1210 leukaemia were cured using mitoxantrone in an IP-IP regime, no long term survivors (0/8) were seen with IV-IV regime.

One possible explanation for the decreased effect in the IV-IP regime is that mitoxantrone is sequestered in the peritoneal tissue compartment and only slowly released (Blochl-Daum, 1988). Thus, the amount of drug reaching the leukaemia was not sufficient to prevent leukaemic growth. Not all drugs are affected by the schedule of administration. Thus, the route of administration of cyclophosphamide (i.e I.V or I.P) did not affect treatment outcome using BNML leukaemia (Hagenbeek and Martens, 1982) unlike what was observed with mitoxantrone in P388 leukaemia (Baguley and Wilson, 1987). Clinically, however, mitoxantrone is usually administered intravenously in the treatment of acute myeloid leukaemia (Alberts et al, 1988).

There was an incidence of leukaemia relapse in this study, occurring 3 months following treatment of low cell dose transplant of SA7 cell line with mitoxantrone (1.5mg/Kg , Table 3). One of the two mice surviving for 90 days was discovered to have enlarged spleen upon autopsy and probably would have died from leukaemia a few days later. This highlights the importance of autopsying all long term survivors(cures) following chemotherapeutic treatment. The central nervous system is an important site of leukaemia involvement although this is more common in acute lymphocytic leukaemia (ALL). Furthermore, the blood-brain barrier may shelter leukaemia cells from systemic chemotherapy. In humans, involvement of CNS is uncommon at diagnosis but it is a frequent site of relapse (Bleyer and Poplack, 1985). CNS relapse is often followed by systemic relapse.

All other autopsied long term (60 days or 90 days) survivors had normal spleen weight, femur cellularity, PCV, Hb and differential bone marrow counts. Even clinically, remission documentation is normally by morphological examination of the bone marrow and a level of 5-10% blasts are thought to indicate remission (Katz et al, 1989). Whether these

blasts are normal regenerating precursors or leukaemic 'rebound' cannot be ascertained by morphology alone. The use of 60 day-survival as a criteria for cure is adopted by many investigators including Fujimoto and Ogawa, 1982; Shenkenberg and Von Hoff, 1986. Similarly, in this study, no difference in number of colony forming cells per $5x10^4$ bone marrow cells was found between normal and cured mice. And recovery of colony numbers has been used as an indicator for haematological remission by Spitzer et al, 1977. Remission colonies in the report exhibited nonleukaemic karyotype.

A lot of factors determine drug response in tumour-bearing mice apart from the drug dose and route of administration discussed above. For example, although all mice used were genetically identical and of the same sex and weight, yet there was marked difference in the response of individual mice to the drug. Some mice were cured (one per dose) while others only had their life-span extended. All mice were injected with the same single cell suspension and by the same individual, yet it is very likely that the leukaemic cell dose received by each mouse was different. It was reported that the subpopulation of tumour cells present in any two experimental animal hosts inoculated with the same tumour will inevitably be different (Poste, 1986). In addition, although the response of males and female mice to mitoxantrone are similar, females seem slightly more sensitive to the per-acute and delayed toxic effects of mitoxantrone than males (Johnson et al, 1979).

One obvious advantage of the SA7 leukaemic cell line (and possibly other models as well) is the fact that it is non-immunogenic (Hepburn et al, 1987). It has been reported that in most human tumours there is no unequivocal evidence that host immunity plays a part in response to therapy. This supports the suitability of non-immunogenic models for experimental chemotherapy studies (Hewit, 1978). However, because no

single tumour model is perfect, it has been suggested that more than a single tumour system should be employed for preclinical drug screening (Kallman et al, 1985). Generally, experimental leukaemia models have proved their value in predicting response in humans. For example, compounds such as epirubicin and idarubicin that showed activity at least equal to doxorubicin in mice were found to be active antileukaemic agents in patients. Anthracyclines that were less toxic (eg aclarubicin) than doxorubicin in mice were also less toxic in patients. And those that were more toxic than doxorubicin (eg carubicin, idarubicin) were also more toxic in man (Casazza, 1986). However, caution must be exercised as not all findings in the laboratory can be directly extrapolated to the clinical situation. For example, humans and mice metabolize epirubicin differently (Casazza, 1986). In addition to the clinical correlations often observed between laboratory models and human patients murine leukaemia models also provide relatively economical preclinical screens (Baumgart et al, 1988).

During exponential growth, both P388 and L1210 lymphoid leukaemias showed a growth fraction of unity (Clausen, Bolstad and Mjelva, 1988). This suggests that all tumour cells present are stem cells. However, this is not the case with human tumours (Mackillop et al, 1983a). Rapidly proliferating tumours are usually more susceptible to drugs than slow growing tumours. This suggests that these models may not be adequate for preclinical screening of phase-specific agents (Aherne, CampleJohn and Wright, 1977). However, similar to the finding in humans, late stage leukaemic disease in LBN-ML model is not curable by even massive doses of Ara-C (Vaughan and Burke, 1983).

In addition to P388 and L1210 leukaemia models, human tumour xenografts are also used in preclinical drug screening. They are attractive because of their clinical origins and there is evidence that they

may retain the therapeutic sensitivity of the source human tumour (Kallman et al , 1985).

6.10 Growth Factor Responses of Recurrent Leukaemias

The SA7 high cell dose transplant leukaemic cell line normally responds to growth factors (WEHi, L929, WEHi+L929) in vitro by increased proliferation that was growth factor concentration-dependant. However, at relapse following (in vivo) treatment with mitoxantrone the leukaemic cells were not responsive to WEHi, L929 or combinations of the two. By the time recurrent disease is diagnosed in humans, the tumour cells present at presentation may be phenotypically very different from those in the original tumour due to the emergence of new subpopulation (Poste, 1986). And in a substantial number of cases, there is a change in French-American-British (FAB) subgroup at relapse (Vanderweide, VanRhenen and Langenhuijsen, 1985). It has been suggested that because many cytotoxic agents (including mitoxantrone) are mutagens, the emergence of new cell variants following treatment is facilitated (Kerbel and Davies, 1982).

When mitoxantrone was administered to leukaemic mice either 24 hours or 48 hours before they became moribund with the disease, the leukaemic cells still retained growth factor sensitivity in vitro. This suggests that the leukaemic cells probably needed to undergo several cell divisions in order to lose growth factor sensitivity. As the leukaemia was virtually established when the drug was administered, the growth rate was decreased. Alternatively, a metabolite of mitoxantrone could be responsible for inducing the leukaemic cells to develop growth factor insensitivity in vitro. It is also unlikely that the observed growth factor insensitivity was due to leaching out of mitoxantrone from the

leukaemic cells which in turn inhibits their growth in vitro. The loss of growth factor sensitivity may be due to loss of receptors for growth factors on the leukaemic cells. For example, it has been reported that adriamycin is capable of regulating the number of epidermal growth factor (EGF) receptors in both Hela and 3T3 cells. The effect requires the continuous presence of the drug for several days (Zuckier and Tritton, 1983). This is probably and adaptive response to anthracycline toxicity (Vichi and Tritton, 1989). Similarly, it has been suggested by Nowell, 1986, that some tumours may have altered response to circulating hormones through loss of specific receptors. The hyporesponsiveness of some autoimmune deficiency syndrome (AIDS) patients to interferon alpha (a-IFN) therapy may be due to decreased numbers of IFN alpha receptors expressed on the cell surface as a consequence of continuous in vivo exposure to endogenous IFN (Lau, Read and Williams, 1988).

Bone marrow cells from normal mice retain their sensitivity to WEHi, L929 and combinations of the two following treatment with mitoxantrone. Similarly, bone marrow cells of mice cured of leukaemia by mitoxantrone treatment were fully responsive to growth factors. Even bone marrow cells of a leukaemic mouse (with no evidence of 'clinical' leukaemia) that died in remission as a result of drug related toxicity 30 days following treatment, retained growth factor sensitivity. Thus only relapsed leukaemic cells showed growth factor insensitivity.

Untreated (SA7HD) leukaemic bone marrow cells formed colonies in culture. In contrast, recurrent leukaemic cells formed very few colonies in culture. Even these may be due to "passenger" normal colony forming cells, although there is no evidence from this study to support such speculation. However, recurrent leukaemic bone marrow cells did not inhibit the growth of normal bone marrow cells when the two were co-cultured together. This is interpreted to mean that although the

recurrent leukaemic cells themselves were insensitive to growth factors and would therefore not form colonies in vitro, the few normal clonogenic cells present in the cell suspension could form colonies. Similarly, it was reported that conditioned medium from human leukaemias that formed colonies in vitro had least inhibitory effect on the growth of normal bone marrow (Gordon, Douglas and Blackett 1978). A survey of the literature did not provide definite explanation for the lack of growth factor sensitivity by relapsed leukaemic cells. In addition, no data from this study is available as explanation for the observed effect. So one can only speculate as to the possible reason(s) for the loss of growth factor sensitivity by recurrent leukaemic bone marrow cells.

The colony stimulating factors (CSF) present in the conditioned media employed (eg IL-3) are necessary for the proliferation of leukaemic cells in vitro. Withdrawal of CSF from haematopoietic cells in vitro is usually followed by rapid cessation of DNA and protein synthesis and cell death (Metcalf, 1985). IL-3 regulates the entry of D-glucose into cells and its presence is necessary for the maintenance of cytoplasmic concentrations of ATP (Dexter, Whetton and Heyworth,1986). This suggests that the recurrent leukaemic cells could be dying in culture as a result of the absence of the above-mentioned processes. The exact mechanisms whereby growth factors trigger cell proliferation is yet unknown. However, autophosphorylation of M-CSF receptor has been noted following binding of M-CSF and the CSFS have been shown to induce changes in the rate of synthesis of a number of cytoplasmic and nuclear proteins (Metcalf, 1985). At the nuclear level, growth factors induce changes in the transcriptional programme of the cell. They induce the expression of nuclear proto-oncogenes C-fos and C-myc . The

product of these proto-oncogenes are believed to be part of the genetic elements involved in cell proliferation (Farrar et al , 1988).

Before growth factors can exert their effect they must first bind to receptors present on the cell membrane. And it has been reported that the binding of growth factors to their receptors appears to be potential target for disruption by cytotoxic agents (Zuckier and Tritton, 1983). It has also been shown that the cell membrane of leukaemic cells could undergo phenotypic shift at relapse. This has been attributed to the effects of chemotherapy (Borella, Casper and Lauer, 1979). Adriamycin could be cytotoxic to L1210 without even entering the cells, probably due to an effect on the cell membrane (Tritton and Yee, 1982).

The most substantial evidence for the possible involvement of receptor loss in the development of growth factor insensitivity by recurrent leukaemic cells, comes from the study of the effect of mitoxantrone on EGF receptors in breast cancer cell line (MCF-7)). After 12-hour incubation with mitoxantrone, EGF binding was dose-dependently decreased. This was due to loss of receptor affinity (Trijssnaar et al, 1989). Similarly, it has been suggested that although the pathways must ultimately diverge, cytotoxic drugs and growth factors may have shared underlying mechanisms (Vichi and Tritton, 1989).

The relapsed leukaemic cells probably modified their growth factor sensitivity in vivo as it enabled the cells to survive the cytotoxic effect of mitoxantrone. The in vivo environment is probably necessary, because the SA7 leukaemic bone marrow cells did not lose growth factor sensitivity, when they were treated with mitoxantrone in vitro. Thus, there appears to be a relationship between drug sensitivity and growth factor response. Studies have shown that AML clonogenic cells from a proportion of patients may be less sensitive as compared to normal granulocyte-macrophage progenitor cells to colony stimulating activity.

The degree of reduction in sensitivity is closely related to the proliferative capacity of the AML colony forming cells in vitro (Francis, 1981). The degree of sensitivity to colony stimulating activity (CSA) at presentation was found to be closely correlated to the response to induction chemotherapy in 15 patients. AML cells that were very insensitive to CSA were associated with a slower response to therapy and a reduced remission rate. Such insensitive cells are unlikely to be in DNA synthesis and could thus provide a more difficult target for cytotoxic agents (Francis, 1981). An alternative explanation may be that all the recurrent leukaemic cells are in G_O state and would therefore not respond to growth factors by increase in proliferation. However, this is unlikely as the leukaemia grows in vivo.

The loss of growth factor sensitivity was also dependent on the route of leukaemia administration similar to what was observed with drug therapy. Thus, minimal growth factor sensitivity was retained when the leukaemic cells were injected I.V with the drug administered I.P. Because with I.P administration, mitoxantrone is sequestered in the peritoneal tissue, little of the drug was available to the leukaemic cells to pose a "threat" to their existence and so make them lose substantial growth factor sensitivity.

Contact inhibition is one of the mechanisms postulated to explain the suppression of normal haemopoiesis in leukaemia (Nara et al, 1984). In addition, a leukaemia inhibitory activity (L1A) has also been implicated (Broxmeyer et al, 1978). Inhibition of colony formation was found when normal marrow was cultured in the presence of leukaemic cells in vitro. Similarly, colony forming unit- fibroblastoid (CFU-F), was suppressed probably to a greater extent (Nara et al, 1984). However, in this study, no inhibition of normal bone marrow growth was observed in vitro when recurrent leukaemic cells were co-cultured with normal bone marrow

cells. It may be because L1A is lost at 37°C within 5 hours (Broxmeyer et al, 1978) and the experiments were conducted at 37°C. Other workers were also unable to detect inhibition of normal bone marrow growth in vitro by leukaemic cells. They suggested that the inhibitor may be too subtle to be detected in vitro (Nara et al, 1984). Other investigators have noted that conditioned medium from human leukaemic cells that form colonies in vitro was least inhibitory to growth by normal bone marrow cells (Gordon, Douglas and Blackett, 1978).

6.10.1 Passage of Recurrent Leukaemic Cells in Mitoxantrone Pretreated Mice

When recurrent leukaemic cells were passaged in mice pretreated with a single dose of mitoxantrone, the resulting leukaemia (called second recurrent leukaemia or PASS MIT 2d) was not responsive to growth factors. In contrast, when the same recurrent leukaemic cells were passaged in mice pretreated 50 days previously, the leukaemia in 2 of 3 mice did not respond to growth factors. The most likely explanation for this difference between the latter mice may be found in the pharmacokinetics of mitoxantrone. Up to 50% of administered dose of mitoxantrone was present in rats 10 days after the drug's administration. Similarly, persistently high levels of ^{14}C-mitoxantrone were found in tissues after dosing (James et al, 1983). And mitoxantrone can still be detected for up to 73 days following dosing. Thus, it is likely that the drug was present in the mice 50 days after it was administered. The reason why the leukaemic cells were growth factor sensitive in vitro in one case may be due to variation in the mitoxantrone metabolism and excretion which occurs even in inbred animals (Hagenbeek and Martens, 1982). Thus, there may be a concentration of mitoxantrone above which the leukaemias need to maintain growth factor

insensitivity to survive and below which they could revert to normalcy. And in the third mouse (M3) , virtually all the mitoxantrone was excreted (within fifty days), so that the net effect of transplantation would be similar to passaging the recurrent leukaemic cells in untreated normal mice.

6.10.2 Passage of Recurrent Leukaemic Cells in Normal (untreated) Mice

When recurrent leukaemic cells were passaged in normal (untreated) mice they regained their growth factor sensitivity. This was also true for recurrent leukaemic cells that were first passaged in mitoxantrone pretreated mice. This suggests that the leukaemic cells were responding to environmental influence. If the receptor mechanism proposed is correct, the recurrent leukaemia would be synthesizing new receptors during its growth in untreated normal mice and would therefore respond to growth factors in vitro when tested at presentation. In support of this is the finding that B6SutA cells increase their receptor number for IL-3 from 10,000 to 100,000 in 18 hours at 37°C (Murthy, et al, 1989). Similarly, it was shown that increase in receptor numbers in Hela and 3T3 cells following treatment with adriamycin involved synthesis of new receptors (Zuckier and Tritton, 1983).

When the recurrent leukaemic cells recovered growth factor sensitivity they also formed colonies in agar. There appears to be a clear cut relationship between exposure of leukaemic cells in vivo to mitoxantrone and loss of growth factor sensitivity monitored using [3H]TdR uptake or colony growth. In this study, there was a close agreement between growth factor sensitivity monitored using [3H]TdR and colony growth of the leukaemia in agar. This suggests that the lack of growth factor sensitivity by recurrent leukaemic cells was not due to an artifact in, say, [3H]TdR uptake determination (eg inhibition of

[3H]TdR transport across cell membrane, competition with endogenous thymidine etc).

6.11 Drug Sensitivity of Recurrent Leukaemic Cells after Passage in Mice

It is well known that tumours that are initially drug sensitive at the beginning of treatment often become progressively less responsive and may ultimately fail to respond during continuing treatment (Schabel et al, 1983). Recurrence in the face of continued treatment usually reflects overgrowth of drug-resistant variants (Griswold, 1986). These are usually detected in patients who relapse after chemotherapy (White et al, 1989). There are relatively few laboratory studies whereby resistance mechanisms are investigated following in vivo treatment. Repeated treatments in vivo mimic the clinical situation better and may prove more applicable to the mechanism of resistance as compared to the usual method of inducing resistance by in vitro treatment (Mattern et al, 1988).

The extent of mitoxantrone resistance developed by recurrent leukaemic cells following passage in normal mice depended on the dose of mitoxantrone that was used in the treatment in vivo. Thus, with very low doses (0.4mg/Kg), the recurrent leukaemic cells were (after one passage in mice) equally sensitive or even slightly more sensitive than untreated leukaemic cells. When 0.75mg/Kg mitoxantrone was administered, the recurrent leukaemic cells were insensitive to low concentrations of mitoxantrone (in vitro) when tested after two passages in normal mice. When mitoxantrone treatment dose was 1.5mg/Kg the degree of resistance increased. The recurrent leukaemic cells (after 2 passages in normal mice) were about 17 times less sensitive as compared to untreated leukaemic cells. With concentrations of mitoxantrone that would inhibit DNA synthesis in untreated leukaemic cells by 80%

relative to control, increased DNA synthesis of the recurrent leukaemic cells was observed. An identical effect was observed in L1210 resistant to Ara-C after the cells were treated with the drug in vitro (Volm, Mass and Mattern, 1980).

Recently, a similar observation was documented clinically. A patient with acute lymphoblastic leukaemia was treated with high dose Ara-C. Following the attainment of remission, the patient relapsed six weeks later. Two cell lines PER-145 and PER-163 were established from bone marrow samples obtained before and after chemotherapy respectively. Exposure of the two cell lines to Ara-C in vitro showed that the primary leukaemia PER -145 was sensitive to Ara-C while PER-163 was resistant. Ara-C concentrations from 1-33μg/ml rather than inhibiting growth (using [3H]TdR uptake assay) actually gave a dose-dependent stimulation of the relapsed cell line (similar to the observation reported in this study). Following subsequent treatment with Ara-C in vivo, the patient's blasts count increased 10 fold, implying that the stimulation observed in vitro probably occurred in vivo as well (Kees, 1987). Similarly, in this study, recurrent leukaemias passaged in mice pretreated with mitoxantrone had bigger spleens as compared to those passaged in normal untreated mice. It may well be that the presence of low concentrations of mitoxantrone in the pretreated mice actually stimulated the leukaemia to divide and hence the increased spleen weight.

The recurrent leukaemic cells probably grow at the same rate (in vivo) as untreated SA7 leukaemia, since no change was observed in the time it takes the recurrent leukaemia to manifest the disease in syngeneic recipients. In one study, no difference in growth rate was seen between Ara-C resistant and sensitive variants of the L1210 leukaemic cell line.

Thus development of drug resistance need not be followed by difference in growth rate (Volm Maas and Mattern, 1980).

A surprising observation was the finding that there was no growth delay when recurrent leukaemic cells were passaged in mitoxantrone pretreated mice. In contrast, mitoxantrone produced significant inhibition of P388 leukaemia growth when administered up to 30 days prior to tumour implantation. Similarly, the degree of prophylactic activity of mitoxantrone against L1210 varied with treatment route, being optimal following I.P administration (White and Durr, 1985).

Another surprising observation regards the in vitro mitoxantrone sensitivity of recurrent leukaemic cells after they were first passaged in mitoxantrone pretreated mice. It would be expected that the degree of mitoxantrone resistance would be increased, but it did not. Recurrent leukaemic cells passaged in mitoxantrone pretreated mice were not more resistant to subsequent treatment with mitoxantrone in vitro as compared to those passaged in untreated mice. Again, it was the dose of mitoxantrone the leukaemic cells were initially exposed to (in vivo), that determines the degree of insensitivity to subsequent mitoxantrone exposure in vitro. The degree of resistance developed by tumour cells may either build up through successive changes or be attained in a single step. The pattern followed is characteristic of the drug. Two patterns have been described: the so-called 'penicillin' pattern in which resistance is reached only through multistep changes; and the 'streptomycin' pattern in which resistance may arise in a single step (Mattern et al, 1988). From this data, it would seem that mitoxantrone mimics the 'streptomcyin' pattern since passaging recurrent leukaemic cells in mitoxantrone pretreated mice did not increase their resistance to mitoxantrone treatment in vitro.

The mechanisms whereby leukaemic cells develop resistance to mitoxantrone is presently unknown. However, it does not appear to involve decreased uptake of the drug into cells although there is a conflicting report. In one study, the uptake of ^{14}C-mitoxantrone was slower in resistant cells than in sensitive cells (Shenkenberg and Von Hoff, 1986). However, Harker et al 1989, reported that mitoxantrone resistant variant of HL-60 cells essentially retains ^{14}C-mitoxantrone to the same extent as the sensitive parental line. In addition, unlike cells which display the classic multi-drug resistant phenotype (MDR-1), mitoxantrone resistant HL-60 cells retained sensitivity to vinca alkaloids, melphalan and mitomycin C. There was also no over expression of p-glycoprotein in resistant HL-60 cells. This suggests that resistance to mitoxantrone is unlikely to be mediated by altered expression of the p-glycoprotein gene. Thus, $Ca^{2\pm}$ channel blocking drug verapamil does not potentiate the cytotoxicity of mitoxantrone in both the sensitive and resistant HL-60 cells. (Harker et al, 1989).

Finally, the differential count of (SA7HD) recurrent leukaemic bone marrow cells was identical to that of untreated SA7HD leukaemic cells. This suggests that the two leukaemias may not be distinguishable morphologically.

6.12 Haematological Toxicity of Mitoxantrone in Normal Mice

In the doses studied (0.4-3mg/Kg) mitoxantrone had no marked effect on blood Hb, PCV and femur cellularity in normal female CBA/H mice. The most noticeable effect was a depression of spleen weight with 3mg/kg and a rise in GM-CFC numbers in vitro with doses in the range 0.4-1.5mg/Kg. The latter effect is probably a compensatory mechanism because the numbers returned to control levels after 16 days. Similarly, increased progenitor numbers were found following treatment of

normal mice with Ara-C (Gherson et al, 1982). A single nontoxic dose of mitoxantrone resulted in extensive destruction of splenic marginal zone cells in rats and mice. It was followed by phagocytosis of cell debris. Small doses gave cumulative effect (Levine and Gherson, 1986). A similar mechanism could be responsible for the decreased spleen weight following treatment of normal mice with 3mg/Kg mitoxantrone.

The limitation of peripheral blood differential count as a determinant of the haematological toxicity of cytotoxic drugs must be borne in mind. In humans, the proliferative ability of bone marrow progenitors measured by colony forming cell assays can be depressed for long periods after exposure to cytotoxic chemotherapy despite normal counts in peripheral blood (Rosenberg et al, 1988). In addition, significant differences exist between mice and humans for direct extrapolation of laboratory to the clinical situation. For example, both human and mouse cells have similar sensitivities to BCNU. However, human cells seem more sensitive to melphalan as compared to murine cells. The converse seems to be true for nitrogen mustard and 5-fluorouracil sensitivity (Ogawa, Bersagel and McCulloch, 1975; Gordon and Blackett, 1976).

6.13 Comparison of Mitoxantrone Sensitivity of Leukaemia-bearing and Normal Mice

In this study, all leukaemia-bearing mice that received 3mg/Kg mitoxantrone died from drug toxicity with no evidence of leukaemia seen in blood film and spleen. However, none of the normal mice that received that dose (3mg/Kg) of mitoxantrone died as a result of drug toxicity in an observation period of 9 days. Most of the leukaemia bearing mice died within 9 days of drug administration. In addition, the femur cellularity in leukaemia bearing mice was depressed following 3mg/Kg mitoxantrone. Apart from the early deaths, some mice

survived for 30 days after treatment only to die from a drug-related toxicity with no evidence of leukaemia. It has been reported that there's a 30-day period usually associated with haematopoietic failure in mice and 75% of deaths occur within that period (Okunewick, Buffo and Kociban, 1985). However, this is unlikely to explain these deaths as the femur cellularity in these mice were hardly affected. It is unlikely to be due to intestinal toxicity either, because these occur within 4-8 days (Okunewick, Buffo and Kociban, 1985). These workers suggested that the haematopoietic toxicity of mitoxantrone may exceed its antileukaemic effect. The myelosuppressive effect of mitoxantrone could be minimized by I.P rather than I.V injection (Alberts et al, 1988).

The parameters that markedly modify the toxicity of antitumour drugs in mice are the strain, the drug vehicle and route of administration. It has been reported that there was a difference of as much as 71% in the LD_{50} obtained by six different laboratories that administered injection of the same drug. Moreover, there was 20% chance with I.P administration that part of the drug was not injected into the peritoneal cavity (Guarino et al, 1979).

All SJL/J normal mice that received mitoxantrone 20mg/Kg were alive up to 8 days after injection. And 60% of the mice that received 10mg/Kg were alive by 90 days (Okunewick, Buffo and Kociban, 1985). Although a different strain of mice (CBA/H) were used in this study, it is still unlikely that 3mg/Kg would be fatal to these mice. Furthermore, doses greater than 142mg/Kg (I.V) were required to produce per-acute lethality in CD_2F1 male mice (Johnson et al, 1979).

Tumour bearing mice were more susceptible to the toxic side effects of Ara-C than tumour free animals. The tumour itself increased the vulnerability of normal cells to the drug (Schafer and Maurer-schultze, 1987). Similarly, there was a clear cut difference as regards the LD_{50} for

cyclophosphamide between normal and leukaemic rats (164 and 110mg/kg respectively). In BNML leukaemic rats, excessive leukaemic cell kill leading to tumour cell embolism in association with impaired functions of vital organs by leukaemic cells were additional factors contributing to early death (Hagenbeek and Martens, 1982). There is evidence that the pharmacokinetics of cytotoxic agents may be influenced by the presence of tumour. Drugs affected included cyclophosphamide, adriamycin and bleomycin. The factors responsible for this effect include reduced metabolic capacity of the liver, impaired kidney function, anaemia, leucocytosis, changes in serum protein contents or toxic factors produced by the tumour. For example, there were significant differences between tumour free mice and mice with leukaemic cell load with respect to the distribution pattern of ^{14}C-ambazone (Kuhnel et al, 1988).

In this study, there was a very marked difference in the sensitivity to toxic effects of mitoxantrone between normal mice and leukaemia bearing mice. It has been reported that coagulation cascade may be activated following treatment of acute promyelocytic leukaemia with antineoplastic agents. This is as a result of release of proagulant activity from the leukaemic promyelocytes and it could lead to disseminated intravascular coagulation (DIC) (Gale, 1985). Similar effect could explain the death of leukaemia bearing mice that were treated with 3mg/Kg mitoxantrone.

6.14 Mitoxantrone Sensitivity of Bone Marrow Cells from Treated Normal and Cured Mice

Reduced sensitivity to mitoxantrone in vitro was displayed by bone marrow cells from all normal mice that received low doses (0.4-0.75mg/Kg) of mitoxantrone. Similarly bone marrow cells of cured mice

exhibited this protective effect. In this case the effect was present no matter what dose of mitoxantrone was used in the treatment of the leukaemia. Thus, although 2mg/Kg administered to normal mice resulted in the loss of protective effect, the bone marrow cells of mice cured of leukaemia with that dose still exhibited this effect. Doses of mitoxantrone that resulted in loss of protective effect by normal bone marrow cells was also toxic to leukaemic mice (eg 2 and 3mg/Kg). No protective effect was seen in bone marrow cells of a mouse dying as a result of drug toxicity. The protective effect was manifested by 10-fold increase in the $I.C_{50}$ of mitoxantrone. And the effect was detected up to 60 days following mitoxantrone administration and was also shown by myeloid progenitor cells as well. Administration of priming doses of antitumour agents can protect animals from subsequent toxic doses of drugs. This protective effect is associated with an increase in glutathione-S-transferase levels in bone marrow cells (Cowan, 1989). It was found that following cyclophosphamide administration, glutathione (GSH) levels were particularly increased in granulocytes. Similar changes were observed following administration of Ara-C in vivo in CBA mice. It was proposed that high GSH and GSH-transferase represent a general response to cytotoxic insult. This provides at least partial explanation for the protection conferred against subsequent toxin administration (Adams, Carmichael and Wolf, 1985). In addition, mitoxantrone is also metabolized by GSH-transferase. These enzymes catalyse the conjugation of thiol containing GSH with electrophillic substrates. They are therefore well suited to protect cells from toxins. GSH levels were elevated for up to 25 months following treatment of patients with alkylating agents (Britten and Green, 1989). These evidences suggest that the protective effect developed by bone marrow cells of cured mice and normal mice treated with low does of

mitoxantrone may be due to the elevation of GSH and or GSH-S-transferase (GST) levels. The ability to develop this protective effect appears to be associated with long term survival in leukaemic mice.

In conclusion, these leukaemic models appear to be good models for studying the cytotoxic activity of antileukaemic drugs. They could be used in the preclinical screening of new drugs or in the re-evaluation of drugs already in clinical use. The development of growth factor insensitivity by relapsed leukaemic cells clearly needs further investigation. It should not only provide clues on the behaviour of leukaemic cells, but could shed light on drug resistance mechanisms.

SUMMARY

1. The SA2, SA7HD and SA8HD myeloid leukaemic cell lines seem equally responsive to cytosine arabinoside (Ara-C) when response was monitored using DiSC assay. However, the SA7HD cell line seem more sensitive to mitoxantrone as compared to SA2 and SA8HD cell lines using this assay. The DiSC assay appears to be simple and convenient although scoring of slides may be subjective.

2. When [3H]-thymidine uptake assay was employed to assess cytotoxicity, the SA2 leukaemic cell line seem most sensitive to DNA synthesis inhibition by mitoxantrone. With the exception of this cell line, neither Ara-C nor mitoxantrone showed differential cytotoxicity against leukaemic cell lines as compared to normal bone marrow cells. In all the leukaemic cell lines studied, mitoxantrone seem more potent than Ara-C, using comparable doses in vitro. However, mitoxantrone was also more toxic to normal bone marrow cells. In the 81287 cell line and its transplant progeny, spleen cells seem slightly more sensitive to Ara-C than bone marrow cells. The primary leukaemic cell line (81287) appears to become less sensitive to both Ara-C and mitoxantrone with passaging. Generally, combinations of Ara-C and mitoxantrone resulted in additive effects. The SA7HD cell line and normal bone marrow cells (NBM) seem susceptible to the effect of drug scheduling (FIGURE S1). In both cases, one hour-pulse with Ara-C followed by the addition of mitoxantrone after 24 hours seem more cytotoxic as compared to when mitoxantrone was added immediately following one hour pulse with Ara-C. The SA2 and SA8 cell lines were unaffected by drug scheduling (FIGURE S1).

3. The SA7HD cell line appears to grow exponentially in culture as indicated by labelling index determination. Mitoxantrone suppressed labelling indices of SA2, SA7HD and SA8HD cell lines in a dose-dependent manner. There was a correlation between in vitro labelling index suppression using high concentration of mitoxantrone, and inhibition of [3H]TdR uptake determined using scintillation counting. However, the autoradiograph method probably underestimates cytotoxicity produced by low concentrations of mitoxantrone.

4. Mitoxantrone was markedly more toxic to normal myeloid progenitor cells as compared to Ara-C when response was monitored using GM-CFC assay. Unlike mitoxantrone, one hour pulse with Ara-C was non-toxic to myeloid progenitor cells in the doses studied.

5. The primary (81287) and low cell dose transplant cell lines (SA7FT5 and SA8FT14) were less responsive to growth factors in vitro as compared to high cell dose transplants of SA7 and SA8 cell lines (SA7HD; SA8HD). The SA2 leukaemic cell line grows in vitro without requirement for exogenous supply of growth factors. However, in serum-free medium, no in vitro growth was observed unless growth factors were added. This strongly suggests that serum was providing the stimulus for the in vitro proliferation of the leukaemic cells. Transferrin alone or saturated with iron, did not stimulate the SA2 leukaemic cells to proliferate in vitro unless growth factors were added as well. L929 conditioned medium in concentration between 5-20% decreases the in vitro proliferation of SA2 cell line. Similarly, 20% WEHi conditioned medium has the same effect. However, when combinations of WEHi and L929 (5-20%) were used, synergistic proliferative response was observed.

6. Mitoxantrone possess antileukaemic activity against both low cell dose transplant and high cell dose transplant of SA7 leukaemic cell line in vivo. A narrow therapeutic margin was observed. The route of administration of leukaemic cells had an effect on therapeutic outcome. Mitoxantrone seem more effective in the high cell dose transplant cell line when both leukaemia and drug were administered intraperitoneally (I.P). The responses of these myeloid leukaemias were distinctly different from those reported in the literature for murine lymphoid leukaemias. For example, doubling the dose of mitoxantrone within the therapeutic range was not accompanied by an increase in the number of long-term survivors (cures). Of the two variants of SA7 cell line studied, the low cell dose transplant appears to be more sensitive to mitoxantrone in vivo.

7. SA7HD leukaemic bone marrow cells were normally responsive to stimulation of proliferation by growth factors(WEHi, L929, WEHi+L929) in vitro. However, in mice with relapsed leukaemia following in vivo treatment with mitoxantrone, the recurrent leukaemic bone marrow cells became significantly insensitive to growth factors in vitro. No growth factor insensitivity was developed by bone marrow cells of normal mice treated with mitoxantrone in vivo. Similarly, bone marrow cells of mice cured of leukaemia by mitoxantrone treatment also responded to growth factors. Furthermore, when mitoxantrone was administered to leukaemic mice 24 or 48 hours before they became moribund with leukaemia, the leukaemic cells still retained growth factor sensitivity. Even bone marrow cells of a leukaemic mouse dying from drug toxicity while in remission, responded to growth factors. When mice inoculated with leukaemia intravenously, were treated

with mitoxantrone intraperitoneally, the resulting recurrent leukaemia retained minimal growth factor sensitivity in vitro , thus, showing route of administration dependence as was observed with drug treatment. The recurrent leukaemic cells did not inhibit the growth of normal bone marrow cells when the two were co-cultured in vitro (FIGURE S2) .

Recovery of growth factor sensitivity was observed upon passaging the recurrent leukaemic cells in normal (untreated) mice. The degree of recovery of growth factor sensitivity appears to depend on the number of leukaemic cells injected prior to treatment. When recurrent leukaemic cells were passaged in mitoxantrone pre-treated mice, no recovery of growth factor sensitivity was observed in most cases (FIGURE S2).

8. Despite recovery of growth factor responsiveness, the sensitivity of recurrent leukaemic cells to subsequent mitoxantrone treatment in vitro depended on the dose of mitoxantrone used in treating the leukaemic mice (in vivo). When a low dose of mitoxantrone (0.4mg/Kg) was used, the recurrent leukaemic cells (after a single passage in mice), were as sensitive as untreated leukaemic cells to mitoxantrone in vitro. However, with a dose within the therapeutic range (1.5mg/Kg), the recurrent leukaemic cells were significantly less sensitive to some concentrations of mitoxantrone in vitro. Passaging the recurrent leukaemic cells in mitoxantrone pre-treated mice did not increase their level of resistance to mitoxantrone. This suggests that the initial mitoxantrone dose administered during treatment was an important determinant of degree of resistance.

No difference in in vivo growth rate was observed between relapsed and untreated leukaemic cells when both were injected in syngeneic

recipients. Similarly, passaging recurrent leukaemic cells in mitoxantrone pre-treated mice was not accompanied by delay in growth of the leukaemia.

9. Normal CBA/H mice seem less sensitive to toxic effects of mitoxantrone as compared to leukaemia bearing mice , inspite of the fact that mitoxantrone treatment was started a day after tumour inoculation, when virtually few or no leukaemic cells were in the bone marrow. This suggests that some mechanisms , other than those associated with leukaemic infiltration, may be responsible for these deaths.

10. Bone marrow cells of cured mice and normal mice treated with low doses of mitoxantrone developed protective effect towards subsequent mitoxantrone exposure in vitro. The protective effect was manifested as a 10-fold increase in mitoxantrone $I.C_{50}$ in vitro which was observed using both [3H]thymidine uptake assay and GM-CFC assay. Bone marrow cells of a mouse dying as a result of drug toxicity while still in remission, did not exhibit protective effect towards mitoxantrone exposure in vitro. Similarly, doses of mitoxantrone that resulted in loss of protective effect by bone marrow cells of normal mice, were also toxic to leukaemia bearing mice. For example,when mitoxantrone (3mg/Kg) was administered to normal mice, the bone marrow cells did not develop protective effect . Similarly, all leukaemia bearing mice that received 3mg/Kg of mitoxantrone, died from drug toxicity with no evidence of leukaemia found on autopsy.

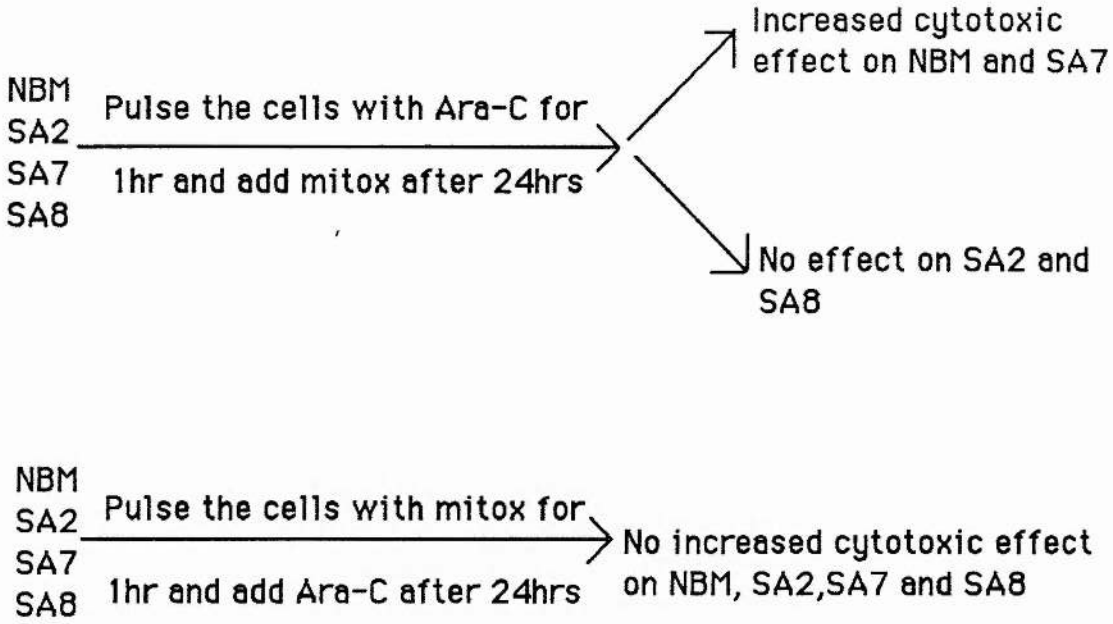
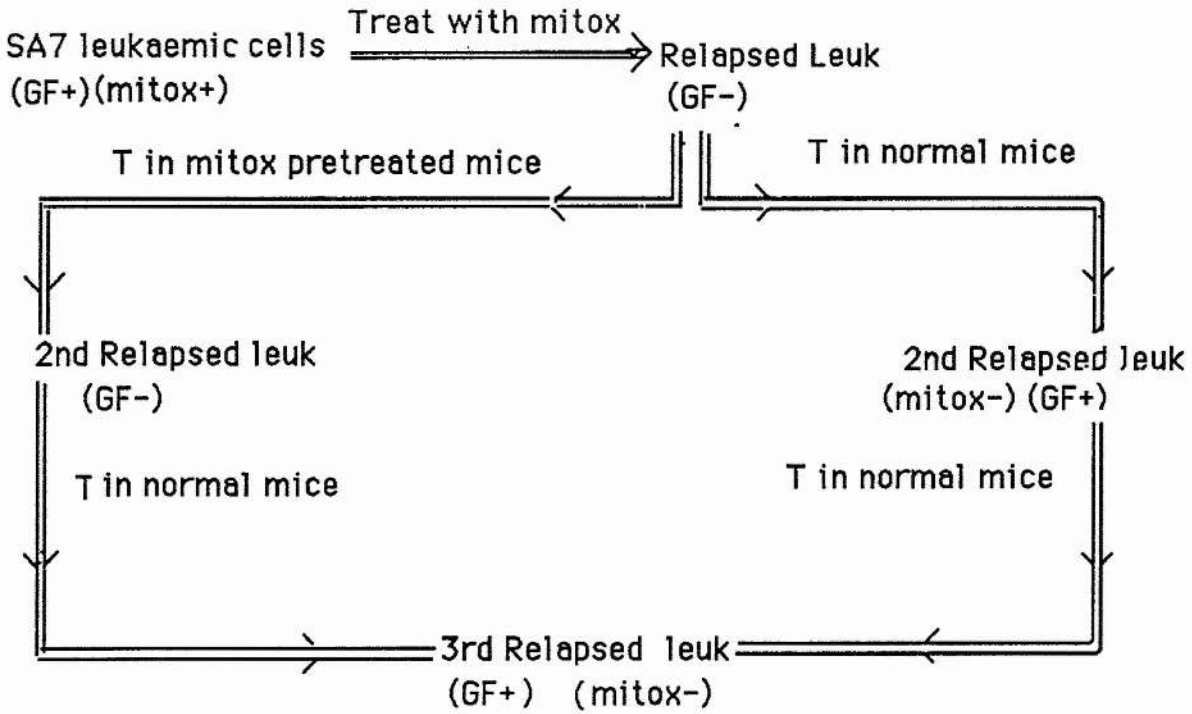


Fig. S1: The effect of drug sheduling on the cytotoxic activity of Ara-C and mitox against NBM cells, and the SA2, SA7,and SA8 leukaemic cell lines monitored in vitro using the [3H]-thymidine uptake assay.



Code:
GF + = Growth factor sensitive
GF - = Growth factor insensitive
mitox + = mitox sensitive
Mitox - = decreased sensitivity to mitox
T = Transplant

Fig.S2: Growth factor (G.F) and mitoxanrone (mitox) sensitivity tests in vitro on relapsed SA7 leukaemic cells following in vivo treatment with mitoxantrone.

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